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Experimental Measurements of Terminal Velocity of Fern Spores

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Abstract.—The dispersal of diaspores is a key process for successful reproduction, survival and evolution of plants. To model the dispersal of biological propagules (e.g., spores, pollen) it is necessary to measure their terminal velocity (V_t) in air. In this study, we used a new method based on video image analysis to measure V_t of spores of seven fern taxa. The average V_t of fern spores was 7.0 cm s^{-1}, but varied among species from 1.6 to 11.3 cm s^{-1}. Similar values were reported previously for airborne particles of mosses and seed plants. Spores of *Danaea nodosa* and *Lophosoria quadripinnata* had a lower V_t than other spores of similar size, perhaps owing to their surface ornamentation that increases their drag coefficient, or an air cavity, which diminishes their density. The reliability of the video image analysis method used here on fern spores was verified by comparing observed and theoretically calculated V_t of glass beads of known diameter and density. This method also allows the observation of spore behavior (e.g., rotation) as they move through the air. Because the method is relatively easy and inexpensive, it can promote future aerobiological research on other biological airborne particles as well.

Key Words.—Aerobiology, settling speed, spore size, spore dispersal

Dispersal is a key process for the successful reproduction, development, and evolution of species. The geographic distribution of plants essentially depends on the dispersal capacity of their propagules and their ability to develop into a

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new reproductive plant under the environmental conditions at the locality where they are deposited (Gagnon, McKindsey, and Johnson, 2015). Ferns are geographically more widespread than angiosperms (Smith, 1972; Tryon, 1970, 1986), probably due to their capacity for long distance spore dispersal (Page, 2002). This enormous dispersal potential is also reflected in their colonization of remote oceanic islands, e.g., they constitute 60% of the flora of Easter Island (Kessler, 2010) and 42% of the flora of Tristan da Cunha (Moran, 2008; Tryon, 1970). On the other hand, it has been reported that most released fern spores are deposited within 10m of the parental plant (Conant, 1978; Peck, Peck, and Farrar, 1990), corresponding to a leptokurtic dispersion (Chung and Chung, 2013). Long distance dispersal of ferns is favored by wind dispersal of single spores followed by gametophytic selfing. Gametophytic selfing, however, reduces genetic variability of new populations. Mechanisms to increase genetic variability at distant locations despite gametophytic selfing were suggested by Haufler *et al.* (2016). Sporophytes produced by gametophytic selfing may survive until the arrival and establishment of a second migrant. Outcrossing between migrants of successional arrival can result in levels of genetic variability comparable to the less likely outcrossing between gametophytes from spores arriving simultaneously and in close proximity (Haufler *et al.*, 2016).

Although a considerable number of aerodynamic studies have measured terminal velocity, size, and density of allergenic spores of fungi and mosses, and pollen and seeds of spermatophytes, these aerodynamic traits have not been evaluated for supposedly innocuous fern spores. However, some recent studies have shown that fern spores may provoke allergic reactions (Chew *et al.*, 2000; de la Cruz, Sánchez-Reyes, and Sánchez-Sánchez, 2011; Kofler *et al.*, 2000) and that spores of *Pteridium* Gled. ex Scop., a highly invasive species, contain a ptaquiloside, a highly carcinogenic compound (Shahin *et al.*, 1998), similar to chemical substances of other fern species (Gomes *et al.*, 2012; Saito *et al.*, 1990).

About 90% of ferns possess an anemochorous syndrome (Sharpe, Mehltreter, and Walker, 2010), i.e. small, wind dispersed, unicellular spores of 20 to 100 μm diameter (Tryon and Lugardon, 1991). Spore size varies depending on their phylogeny and ploidy level (Henry, Bainard, and Newmaster, 2015). Based on their external morphology, spores are monolete (formed by a tetragonal tetrad) or trilete (formed by a tetrahedral tetrad). All spores have a cell wall of sporopollenin, a waterproof and chemically highly resistant polyterpene, which protects the spore from adverse environmental conditions such as UV rays and extreme temperatures during their long trajectories and before they can colonize new habitats (Ballesteros, 2011).

One of the fundamental parameters to measure or model air mediated dispersal is the terminal velocity (V_t), also known as the settling speed or deposition velocity. This is the maximum velocity that a particle achieves when falling through any motionless fluid due to gravity. The V_t depends on characteristics of the particle (density, size) and the fluid (density, viscosity) as resumed by Stokes' law, and was considered as an accurate estimator of pollen

settling rate (Gregory, 1973). In aerobiological science, V_t has been used to develop simple dispersal models assuming that a particle falling vertically at V_t will be displaced horizontally to a distance that depends directly on horizontal wind speed and the height of particle release (Cousens, Dytham, and Law, 2008; Niklas, 1985; Okubo and Levin, 1989; Zotz *et al.*, 2016). More elaborated models, such as the Lagrangian stochastic models, add more environmental parameters (Kuparinen, 2006).

Despite its relevance for particle dispersal, V_t has been measured for only a few pteridophyte spores (Gregory, 1973; Niklas, 1985; Zotz *et al.*, 2016). Moreover, there is no standard methodology to measure V_t. Consequently, reported measurements of V_t of different particles are not easily comparable, especially when they were measured under different conditions. Some researchers estimated V_t by means of different equations (Jiménez and Madsen, 2003). Other authors were improving settling columns with a metric scale to measure settling speed of individual and clumped particles (Aylor, 2002; Di-Giovanni, Kevan, and Nasr, 1995; Ferrandino and Aylor, 1984; Hall and Walter, 2011; Jongejans and Schippers, 1999; Sundberg, 2010) or collected settled particles at each time unit with a mobile rotating disk at the base of the settling column (Borrell, 2011; Sosnoskie *et al.*, 2009). Photographic methods to measure V_t were based on stroboscopic illumination (Green, 1980; Niklas, 1985), or high-speed cameras taking pictures at 15 to 30 frames per second to follow the path of individual particles (Caplat, Nathan, and Buckley, 2012; Loubet *et al.*, 2007; Wright *et al.*, 2008). Even more specialized devices to measure V_t are the Paul trap, which uses electrodes to capture pollen grains, the Millikan chamber (Kohler, Schultz, and Helm, 2007), and laser devices (Agrawal *et al.*, 1996; Askew *et al.*, 1997; Wall, John, and Rodgers, 1985; Zotz *et al.*, 2016). Because the latter devices for accurate measurements of V_t are expensive, data acquisition of aerodynamic properties of a wider array of natural particles was limited. The goals of this study were to describe a new, easy, and inexpensive method to obtain reliable data of V_t of airborne particles and to apply it to measure the terminal velocity (V_t) of spores of seven fern species with different spore characteristics.

Material and Methods

Biological material.—Fresh material of fertile leaves with mature sporangia was collected from a number of selected fern species: *Acrostichum aureum* L., *Cyathea bicrenata* Liebm., *Danaea nodosa* (L.) Sm., *Lophosoria quadripinnata* (J. F. Gmel.) C. Chr., *Onocleopsis hintonii* F. Ballard, *Pteridium caudatum* (L.) Maxon, *Sphaeropteris horrida* Bernh. These species were selected to cover a wide range of spore sizes with diameters of 23 to 73 μm (Tryon and Lugardon, 1991). Spores of *D. nodosa* and *O. hintonii* were monolete, bean-shaped; all other species have trilete, tetrahedral spores. For comparative purposes, pollen grains of *Araucaria heterophylla* (Salisb.) Franco collected at the UAMIZ campus were used to measure the terminal velocity of a gymnosperm. Ferns were collected from natural habitats within Mexico and the material was

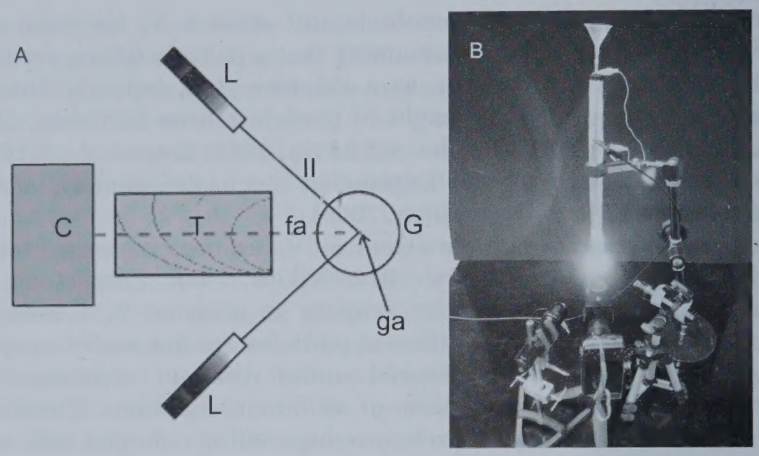

FIG. 1. Settling column to measure terminal velocity. (A) Top view indicates the position of camera (C), telescope (T), two lamps (L) and glass tube (G). The light ray (ll) and the focal axis (fa) are in the same plane and the longitudinal axis of the glass tube (ga) is perpendicular to this plane. (B) The mounted system, connected to physical ground to avoid electric charges.

processed according to Ramírez-Trejo *et al.* (2013), and stored in vials at room temperature until further use.

Particle traits.—The length of the equatorial and polar axes and P:E ratio of 30 spores of each species was measured according to Punt *et al.* (2007). The average mass of fern spores was measured following Gómez-Noguez *et al.* (2016). Some spores were embedded in paraffin, cut (8μm) on a Leica microtome, and placed in fragments of cover slips. After dewaxing, spores were maintained on absolute alcohol and subjected to critical point dehydration in a Leica desiccator (EMCPD 030). They were then mounted on aluminum pins with carbon conductive adhesive tape. In addition, spores were transferred using a brush of few bristles and sprinkled on the sample holder. Both types of samples were covered with a thin layer of gold-palladium by use of a Denton Vacuum desk II ionizer and were observed using a scanning electron microscope (SEM) model Jeol JSM-5310LV.

Terminal velocity of fern spores.—We built a sedimentation tower of 40 cm height using a glass tube (Ø 18 mm), a telescope with ruled eyepiece (100 μm), two sources of light CREE Q5 LED, 3 w, 800 lumens (CREE, INC., DURHAM, LE, USA), and a EXILIM EX-ZR100 camera (CASIO COMPUTER CO. LTD., Tokyo, JAPAN). We aligned the camera and telescope using a CELESTRON universal adapter (Fig. 1). Considering an ideal V_t of 30 cm s^{-1}, the particles reach the maximum speed after 0.071 s at a distance of 1.3 cm (see particle inertia time scale in Alonso and Finn, 1970; Loubet *et al.*, 2007; Vernon and Olsson, 1973).

To avoid electric fields (electric charge accumulation or electric fields due friction), we covered the glass tube with aluminum foil like a Faraday cage, and the entire system was connected to physical earth (Fig. 1 B). The absence

of electric fields in the system was corroborated with an electroscope. All measurements were performed in Mexico City at 2450 m asl, with a gravity of 9.780475 m s^{-2} (CENAM, 2006), a relative humidity of 54%, a temperature of 24°C and an atmospheric pressure of 585 mm Hg. The air density was within the range of 0.770 to 0.916 kg m^{-3}.

To compare observed V_t and predicted V_t by the Stokes' model, we measured the size and V_t of microspherical Ballotini glass beads (Soda lime, ρ=2.6 g cm^{-3}), previously sieved through two phytoplanktonic meshes of 50 and 20 μm to obtain glass beads within this range size. To release individual spores rather than polyads (e.g., clumps of several spores), we attached a regular bevel needle with an inner diameter of 200 μm at the top of the sedimentation tower, which released spores by manual mechanical vibration. We used one needle per species to avoid contamination and washed the glass tube after each species. Videos were filmed with a rate of 1000 fps and analyzed with Tracker video analysis and modeling tool v. 4.87 (http://physlets.org/tracker/). We followed the displacement of the particles frame per frame, graphed distance vs time and used a linear regression model to calculate the particle's terminal velocity (intercept, Figs. 2 A, B). For each species, we analyzed 22 to 130 particle trajectories. Larger particles than those measured previously for each species, and that may represent polyads rather than monads, were excluded.

A Kruskal-Wallis multiple comparison test was performed to detect differences between experimental V_t of species. A t-test was performed to compare between predicted and observed V_t of Ballotini microspheres (control). These analyses were carried out with NCSS statistical software.

Results

Particle size.—Fern spore diameter ranged among species from 23.5 ± 0.42 to 73.2 ± 1.84 μm (Table 1).

Terminal velocity of fern spores.—Mean experimental terminal velocity of fern spores was 7.05 ± 5.35 cm s^{-1} (Table 2), but differed significantly among species ($H=297$, $P<0.0001$). Differences between the slowest and smallest spores of *D. nodosa* and the fastest and largest spores of *L. quadripinnata* were nearly tenfold. Our experimental measurements and video observations also indicated that fern spores of all species were increasing or decreasing the V_t, depending on which part of the spore was facing into the direction of movement. These differences were especially evident on the smallest spores of *D. nodosa* (Fig. 2 B).

There were no significant differences between observed and calculated values of V_t of glass beads, indicating that the measurements using this new method are reliable to determine V_t of these particles ($t = 1.81$, $P = 0.07$). Nevertheless, the V_t of *A. heterophylla* differed ($t = 7.05$, $P < 0.001$) from the mean observed for *A. cunninghamii* Aiton *ex* D. Don, by Hall and Walter (2011).

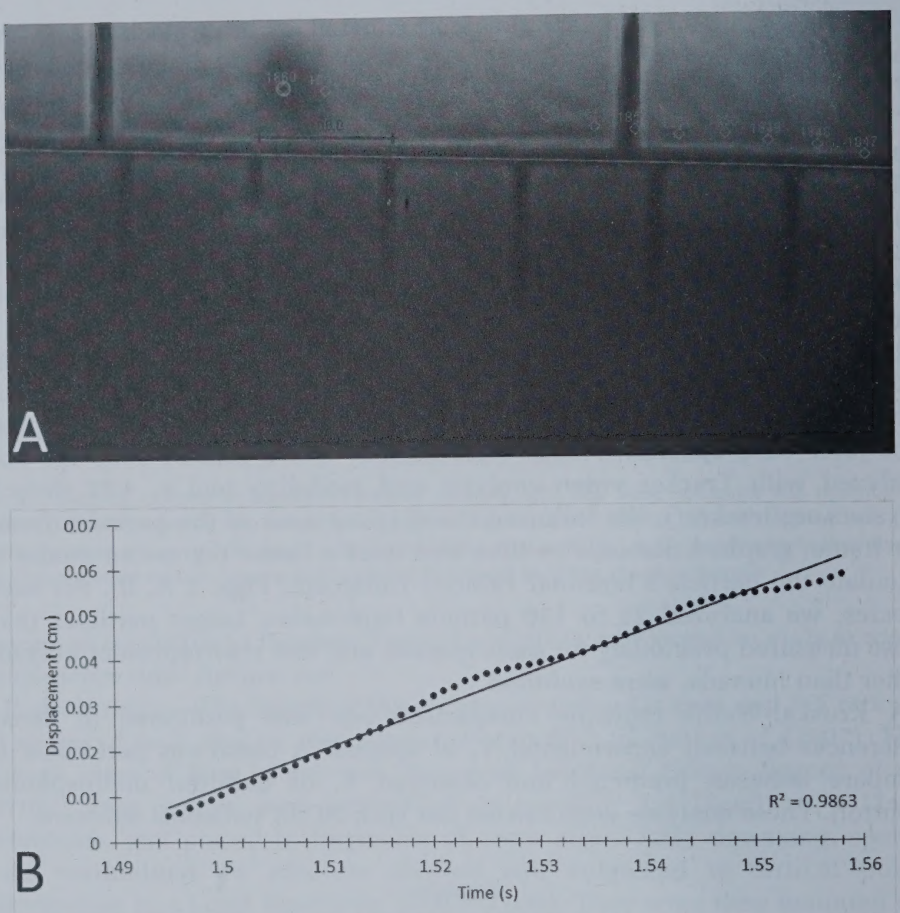

FIG. 2. (A) Picture showing *O. hintonii* spore and previous 14 frames (positions). The y axis is parallel to scale, each bar represents 100 μm. (B) Terminal velocity in air of spores of *Danaea nodosa*. The reniform shape of their monolete spores causes changes in their terminal velocity, depending on the spore orientation relative to the direction of their movement.

TABLE 1. Vouchers, particle size and P:E[1] ratio of fern spores (* gymnosperm pollen), w/n, without number, [1]Polar axis : Ecuatorial axis.

Species	Vouchers (UAMIZ)	Size (μm)	P:E ratio
Danaea nodosa	BPG-1231	23.5±0.42	0.81
Cyathea bicrenata	FGN-302	28.7±0.67	0.70
Sphaeropteris horrida	AMR-1258	32.0±0.69	0.74
Pteridium caudatum	RRT w/n	33.3±0.45	0.88
Acrostichum aureum	FGN-299	52.2±0.80	0.85
Onocleopsis hintonii	FGN-282	57.3±1.6	0.73
Lophosoria quadripinnata	BPG-926	73.2±1.84	0.79
*Araucaria heterophylla**	MMMA-48	72.7±1.46*	0.96

TABLE 2. Terminal velocity of biological particles (fern spores, fungal spores, moss spores, pollen grains, and seeds). n = number of measurements.

Taxa	Terminal velocity (cm s^{-1})
Danaea nodosa, n=130	1.59±0.15*
Cyathea australis	<2[4]
Onocleopsis hintonii, n=49	5.54±0.41*
Polypodium vulgare	6[14]
Cyathea bicrenata, n=22	6.71±0.52*
Pteridium caudatum, n=26	7.91±0.44*
Sphaeropteris horrida, n=22	10.11±0.77*
Acrostichum aureum, n=22	10.87±0.87*
Lophosoria quadripinnata, n=117	11.28±0.75*
Lycopodium L. (lycophyte spore)	2[11]
Stemonitis smithii T. Macbr (slime mold spore)	0.05[10]
Lycoperdon perlatum Pers. (fungal spore)	0.04[10]
Lamproderma sauteri Rostaf (slime mold spore)	0.38[10]
Sphagnum L. (moss spore)	1.3[9]
Pinus taeda L. (gymnosperm pollen)	2.1[12]
Araucaria cunninghamii Aiton *ex* D. Don (gymnosperm pollen)	7.7[4]
Ephedra foeminea Forssk. (gymnosperm pollen)	10.2[2]
Araucaria heterophylla (Salisb.) Franco (gymnosperm pollen) n=46	18.3*
Ambrosia L. (angiosperm pollen)	0.6[8]
Phleum L. (pollen)	1[8]
Cannabis sativa L. (pollen)	2 to 3[11]
Alnus Mill. (pollen)	2 to 3[11]
Corylus avellana L. (pollen)	2 to 3[11]
Glycine max (L.) Merr. (pollen)	2.4[13]
Fagus sylvatica L. (pollen)	5.5[3] to 6[11]
Oryza sativa L. (pollen)	5.8[13]
Zea mays L. (pollen)	24-30[11], 13.6[4]
Stenorrynchos speciosum (Jacq.) Rich (orchid seed)	9[14]
Chamaenerion angustifolium (L.) Scop. (seed)	17[6]
Tragopogon porrifolius L. (seed)	36[6]
Betula populifolia Marshal (seed)	39[6]
Eupatorium rugosum Houtt. (seed)	45[6]
Tragopogon pratensis L. (seed)	55.3[1]
Ailanthus altissima (Mill.) Swingle (seed)	56[6]
Aster prenanthoides Mull. *ex* Willd. (seed)	66[6]
Pinus taeda L. (gymnosperm seed)	70[6]
Acer L. (seed)	76 to 124[6]
Leontodon autumnalis L. (seed)	123[5]
Platanus occidentalis L. (seed)	166[6]
Leucanthemum vulgare Lam. (seed)	262.5[5]
Halesia monticola (Rehder) Sarg. (seed)	338[6]
Silene latifolia Poir. (seed)	435[5]

[1] Askew *et al.*, 1997; [2] Bolinder, Niklas, and Rydin, 2015; [3] Gregory, 1973; [4] Hall and Walter, 2011 (dehydrated); [5] Jongejans and Schippers, 1999; [6] Matlack, 1987; [7] Nathan *et al.*, 2002; [8] Raynor *et al.*, 1966; [9] Sundberg, 2010; [10] Tesmer and Schnittler, 2007; [11] Traverse, 2007; [12] Williams, 2008; [13] Yoshimura, 2011; [14] Zotz *et al.*, 2016; * this study.

Discussion

In this study, we experimentally measured terminal velocity of fern spores. Our results showed a large variation of V_t among fern species with spores of different size and mass, and proved that even very small biological particles such as fern spores may differ considerably in their V_t and consequently in their potential dispersibility. A comparison of their V_t with other biological particles such as fungal spores, pollen grains, and seeds (Table 2) shows that V_t of fern spores is mainly within the range of similar-sized pollen grains, but faster than that of smaller fungal spores and slower than that of most, apparently larger seeds.

The method used here to measure the terminal velocity of fern spores is relatively inexpensive and easy. However, we had to deal with some difficulties. For instance, the focus of the video must be kept on the plane of the particle trajectory, and the high intensity of light required to illuminate particles may cause disturbing light reflections on the surface of the glass tube. The observed minor changes of V_t during the trajectory of a single particle are due to rotational movements of the fern spores, which change the cross–sectional area in the direction of movement and cause increasing or decreasing drag forces (Fig. 2). We assume that the echinate ornamentation of *D. nodosa* spore is responsible for these alterations of the drag forces. A similar movement was observed for spores of *O. hintonii* with extended perispore ornamentation. However, some spores fall without rotational movement, probably due to a more stable center of gravity. For example, *S. horrida* has also echinate but trilete rather than monolete spores, which may stabilize during their trajectory like the NASA space shuttle Galileo Probe Deceleration Module, which is of similar shape (Bienstock, 2004). Spores of *O. hintonii* are of particular interest. Despite their medium size, they possess a low V_t (Table 2, Fig. 4), probably because their wings increase friction (Fig. 3 I).

The reported values of V_t for fern spores of our study are consistently similar to those for conifer and angiosperm pollen grains, with some exceptions. The echinate spores of *D. nodosa* had many spine-like projections of their perispore (see Fig. 3 C-D), which increase their surface and diameter and may consequently decrease wing loading and increase the upwards directed drag, modifying the air flux around the particle. Despite the larger spore size of *L. quadripinnata*, its experimental V_t was comparable to the smaller spores of *S. horrida*. The reduced V_t of *L. quadripinnata* spores may be related to the cingulum (a thick, wing-like projection along the equator of the spore [Punt *et al.*, 2007]) and to their lower density due to the presence of an air-cavity observed in this spore (Gómez-Noguez *et al.* 2016). The cingulum may also produce a combined effect on drag comparable to the thread wake of pollen grains of *Pinus* L. (Grega *et al.* 2013).

Because of pores and apertures in the sporopollenin envelope, most pollen grains undergo a harmomegatic effect, i.e., modifications of their shape by exchanging water with their surrounding medium, and thus, altering their

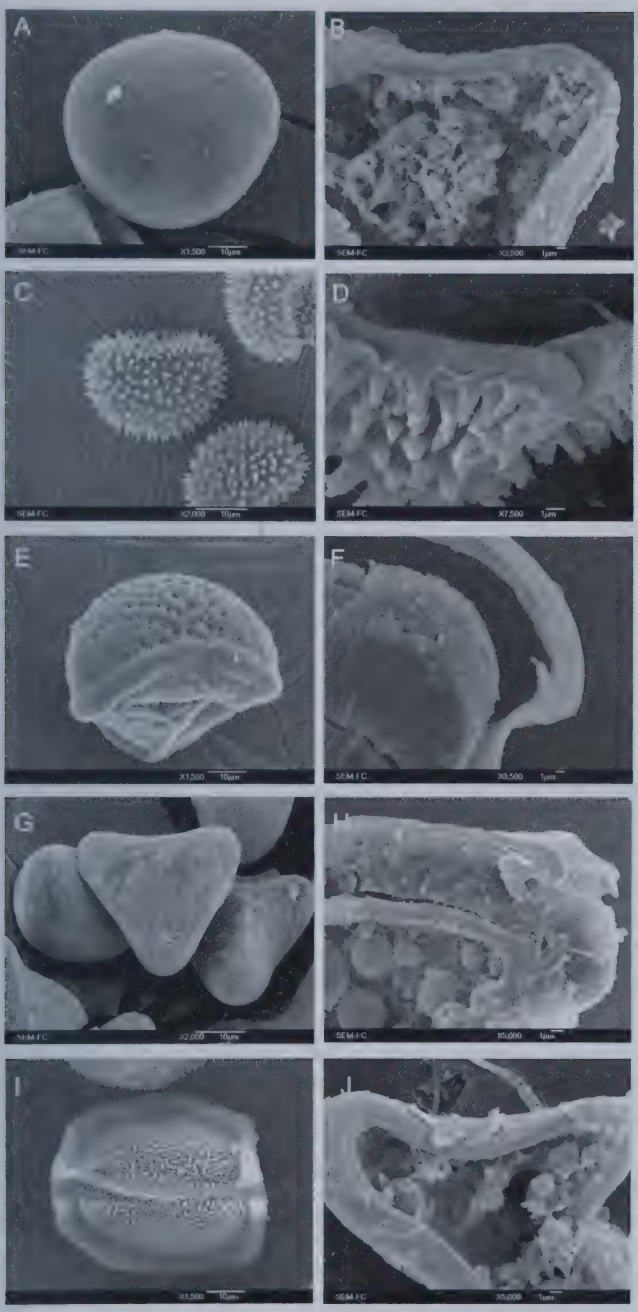

FIG. 3. Spore shape and ornamentation (A, C, E, G, I), and detail of the sporopollenin envelope (B, D, F, H, J) of studied species. (A-B) *Acrostichum aureum*, (C-D) *Danaea nodosa*, (E-F) *Lophosoria quadripinnata*, (G-H) *Cyathea bicrenata*, (I) *Onocleopsis hintonii*, (J) *Sphaeropteris horrida*.

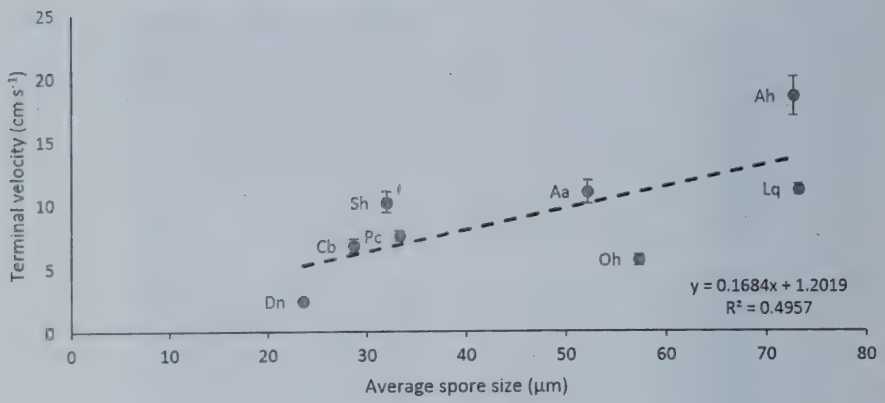

FIG. 4. Terminal velocity of particles ± standard error (fern spores and *Araucaria* pollen grain) sorted by size (μm) and regression line model. Dn=*Danaea nodosa*, Cb=*Cyathea bicrenata*, Sh=*Sphaeropteris horrida*, Pc=*Pteridium caudatum*, Aa=*Acrostichum aureum*, Oh=*Onocleopsis hintonii*, Ah=*Araucaria heterophylla*, Lq=*Lophosoria quadripinnata*.

mass, volume, and density (Hesse, 2009; Volkova, Severova, and Polevova, 2013; Wodehouse, 1935), which increases considerably the variation of measurements of V_t of pollen grains (Hall and Walter, 2011). Such variation could be observed in our measurements of V_t in *A. heterophylla* (54% R H), when compared with *A. cunninghamii* (dehydrated). However, the harmomegatic effect is not expected to be of major importance for fern spores because they do not possess this kind of aperture. Furthermore, fern spores possess a thick and rigid exospore layer (Figs. 3 B, D, F, H, and J) and do not vary much in their water content, even after changes of relative air humidity (Ballesteros and Walters, 2007). Only the cellular substances, particularly the lipid content could affect the V_t by decreasing spore density. Gemmrich (1977) found significant variations in the lipid content of fern spores of different species. These differences may explain why our measurements of V_t of *C. bicrenata* were distinct from those reported for *C. australis* (Hall and Walters, 2011), although the former species had a massive exospore (Fig. 3 H) that may increase spore density and V_t.

The V_t of the fern spores we studied was successfully obtained by use of a new, simple, video analysis method, which will hopefully promote a broader interest in aerobiological research of these extremely diverse biological particles in the future and improve our understanding of the species-specific effect of spore ornamentations on V_t.

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Selaginella hyalogramma (Selaginellaceae – Lycopodiophyta): A New Species from Venezuela, South America

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Abstract.—As part of a revision of *Selaginella* subg. *Heterostachys* in Central and South America, *S. hyalogramma* Valdespino is described as a new species and illustrated with a line drawing, as well as with scanning electron microscope (SEM) images of leaves, sporophylls, and megaspores. This new species is characterized by non-articulate stems, heteromorphic vegetative leaves with idioblasts on their upper and lower surfaces that are also present on sporophylls, as well as dorsiventral, resupinate strobili and a laminar flap on lower surfaces of dorsal sporophylls. These characters serve to compare it to morphologically similar taxa. In addition, the presence of stomata along basiscopic leaf margins and basiscopic submarginal short or tooth-like hairs on upper surfaces of lateral leaves of this species are reported and discussed. Finally, *S. hyalogramma* is only known from a single collection made in the State of Táchira, Venezuela, where it grows as an epipetric plant on mossy sandstone areas that may be severely deforested and, hence, it is tentatively considered Critically Endangered (CR) according to IUCN categories and criteria.

Key Words.—Cerro La Camiri; *Heterostachys*; hairs/trichomes; idioblasts; Parque Nacional El Tamá; resupinate strobili, State of Táchira; submarginal stomata.

Resumen.—Como parte de una revisión de *Selaginella* subg. *Heterostachys* en Centro– y Sur América, *S. hyalogramma* Valdespino se describe como una nueva especie e ilustra con un dibujo, así como con imágenes de microscopía electrónica de barrido (MEB) de las hojas, esporofilos y megasporas. Ésta nueva especie se caracteriza por sus tallos no articulados, hojas vegetativas heteromórficas con idioblastos en sus superficies superiores e inferiores, los cuales también están presentes en los esporofilos, así como por sus estróbilos dorsiventrales y resupinados, y por poseer un ala laminar en la superficie inferior de los esporofilos dorsales. Dichos caracteres sirven para comparar a la nueva especie con otros taxa morfológicamente similares. Adicionalmente, la presencia de estomas a lo largo de los márgenes basiscópicos y de pelos cortos o parecidos a dientes en la superficie superior de las hojas laterales cerca de los márgenes basiscópicos de ésta especie se reportan y discuten. Por último, *S. hyalogramma* sólo se conoce mediante un especimen recolectado en el Estado de Táchira, Venezuela, donde crece como una planta epipétrica en un área de piedras de arenisca cubiertas por musgos, la cual puede estar severamente deforestada, por lo que tentativamente ésta especie se considera En Peligro Crítico (CR), de acuerdo a las categorías y criterios de la UICN.

Palabras Clave.—Cerro La Camiri; estomas submarginales; estróbilos resupinados; *Heterostachys*; idioblastos; Parque Nacional El Tamá; pelos/tricomas; Estado de Táchira.

Over the last quarter of a century, renewed interest in the taxonomy of *Selaginella* P. Beauv. in the Neotropics has led to the description of several taxa (e.g., Mickel and Beitel, 1988; Mickel, J. T., A. R. Smith, and I. A. Valdespino, 2004; Smith, 1990; Valdespino, 1992, 1993, 2015a, b, c, d, 2016; Valdespino, López, and Góes-Neto, 2014, Valdespino *et al.*, 2015), with the

total number of species in the world estimated at 600–750 (PPG I, 2016; Valdespino, 2016 and references therein) or ca. 800 (Zhou and Zhang, 2015). In the New World, there are about 300 *Selaginella* species, of which ca. 270 are found in the Neotropics. Certainly, *Selaginella* is the largest extant lycophyte genus (Valdespino, 2016) and one of the major groups of seedless vascular plants along with the fern genera *Elaphoglossum* Schott ex J. Sm. and *Asplenium* L. (PPG I, 2016).

Selaginella hyalogramma is here validated as the last of the three new species recognized in my doctoral thesis (Valdespino, 1995). This new species has non-articulate stems, heteromorphic vegetative leaves, and dorsiventral strobili composed of dimorphic sporophylls, with the largest sporophylls in the same plane as the median leaves and the smaller sporophylls in the same plane as the lateral leaves (i.e., resupinate strobili). According to the recently proposed subgeneric classification system by Weststrand and Korall (2016a) based on molecular and morphological data (Weststrand and Korall, 2016b), *S. hyalogramma* belong in subg. *Stachygynandrum* (P. Beauv. ex Mirb.) Baker. In Weststrand and Korall (2016a) subg. *Stachygynandrum* is broadly defined to include subg. *Heterostachys* Baker and comprises most heteromorphic leaved, non-articulate species with typically ventral rhizophores, and either quadrangular or dorsiventral (including resupinate) strobili with or without dimorphic sporophylls. Nevertheless, in *Selaginella*, there is a wider morphological and anatomical variation than accounted for by Weststrand and Korall (2016a, b), at least as rhizophores are concerned. One can find, for example, species with only ventral, axillary, or dorsal rhizophores, as well as those with a combination of either two or three kinds of rhizophore position mentioned in the same plant. Likewise, other taxa have lateral rhizophores, usually in combination with the previously described rhizophore positions (e.g., in some fern- and centipede-like species). As noted below, rhizophores in *S. hyalogramma* are axillary, while the strobili are resupinate with the dorsal sporophylls having a distinct laminar flap. Based on those characters, *S. hyalogramma* may be placed in subg. *Heterostachys* Baker, following Jermy's (1986, 1990) morphology-based subgeneric classification. The latter subgenus as emended in Zhou and Zhang's (2015) classification, grounded on both molecular and morphological characters (Zhou *et al.*, 2015), is recognized as monophyletic. Therefore, given that the phylogenetic relationships and classification at the subgeneric level in *Selaginella* are still in flux and for the purpose of future comparative research that better bridge and correlate results from molecular- and morphology-based phylogenetic reconstructions, I tentatively place *S. hyalogramma* in subg. *Heterostachys* Baker, after Jermy (1986, 1990) and Zhou and Zhang (2015).

Selaginella hyalogramma grows at mid elevations on mossy sandstone in forested areas of the state of Táchira, in northwestern Venezuela; a region with a northern Andean flora influence. This country has the highest number of *Selaginella* taxa in the Neotropics with approximately 100 species (Valdespino, 2016).

Material and Methods

This study is based on examination of a single collection comprising two duplicates, one at MO and the other at UC (herbarium acronyms follow Thiers, 2017). Samples of leaves, sporophylls, and megaspores were taken for Scanning Electron Microscopy (SEM) study to document their shape and surfaces, as well as spore ornamentation. The SEM samples were prepared following standard techniques as described by Valdespino (1995) and viewed and photographed at different magnifications using a AMRAY Model 1838 SEM at 10kV. Photographic SEM negatives were later digitized and post-processed with Adobe Photoshop to adjust contrast and make the background black.

The terminology used to describe leaves, sporophylls, and spores, as well as the procedure to measure them follows Valdespino (1995, 2016), Valdespino, López, and Góes-Neto, 2014, and Valdespino *et al.* (2015). Complementary terminology on spore sculpturing patterns derives from Punt *et al.* (2007) and Hesse *et al.* (2009).

Conservation status was assessed according to the IUCN Red list Categories and Criteria version 3.1, second edition (IUCN, 2012).

Taxonomic Treatment

Selaginella hyalogramma Valdespino ***sp. nov.*** Type: VENEZUELA. Táchira: Distrito Córdoba, Cerro La Camiri, [Parque Nacional El Tamá], S of the town of Río Negro, 7°36′00"N, 72°36′00"W, 430–530 m, 6 Nov 1982, *G. Davidse & A.C. González 21525* (holotype: MO barcode MO-255526!; isotype: UC barcode UC1524843!). **Figures 1A–F; 2A–H; 3A–D.**

Selaginella muscosae Spring affinis sed foliis intermediis basi asymmetricis, apice acuminatis diversa.

Plants epipetric. *Stems* ascending to suberect, stramineous to greenish, 4–7 cm long, 0.4–0.6 mm diam., non-articulate, not flagelliform, not stoloniferous, 2- or 3-branched. *Rhizophores* axillary, restricted to the basal ⅓–½ of the stem, filiform, 0.1 to 0.2 mm diam. *Leaves* heteromorphic throughout, membranous, upper and lower surfaces glabrous (except for upper surfaces of lateral leaves near basiscopic margins, where small tooth-like hairs are present), covered by straight-walled, papillate idioblasts on both sides of the midribs (or idioblasts indistinct on lower surfaces of median leaves), the papillae mostly in 1 row over each cell lumen, the upper surfaces green comprising quadrangular to rounded, sinuate-walled cells, many of the cells covered by 1–6 papillae, the lower surfaces silvery green comprising elongate, sinuate-walled, and glabrous cells. *Lateral leaves* distant, almost perpendicular to the stem, broadly ovate to broadly ovate-deltate, 2–2.5 × 1.0–1.5 mm; bases rounded to semicordate, the acroscopic bases strongly overlapping the stems, the basiscopic bases free from the stems; acroscopic margins weakly hyaline, in a band 1 or 2 cells wide with the cells elongate, straight-walled and papillate, parallel to margins, papillae in 1 row over each cell lumen, serrate or shortly ciliate along proximal ¼ and

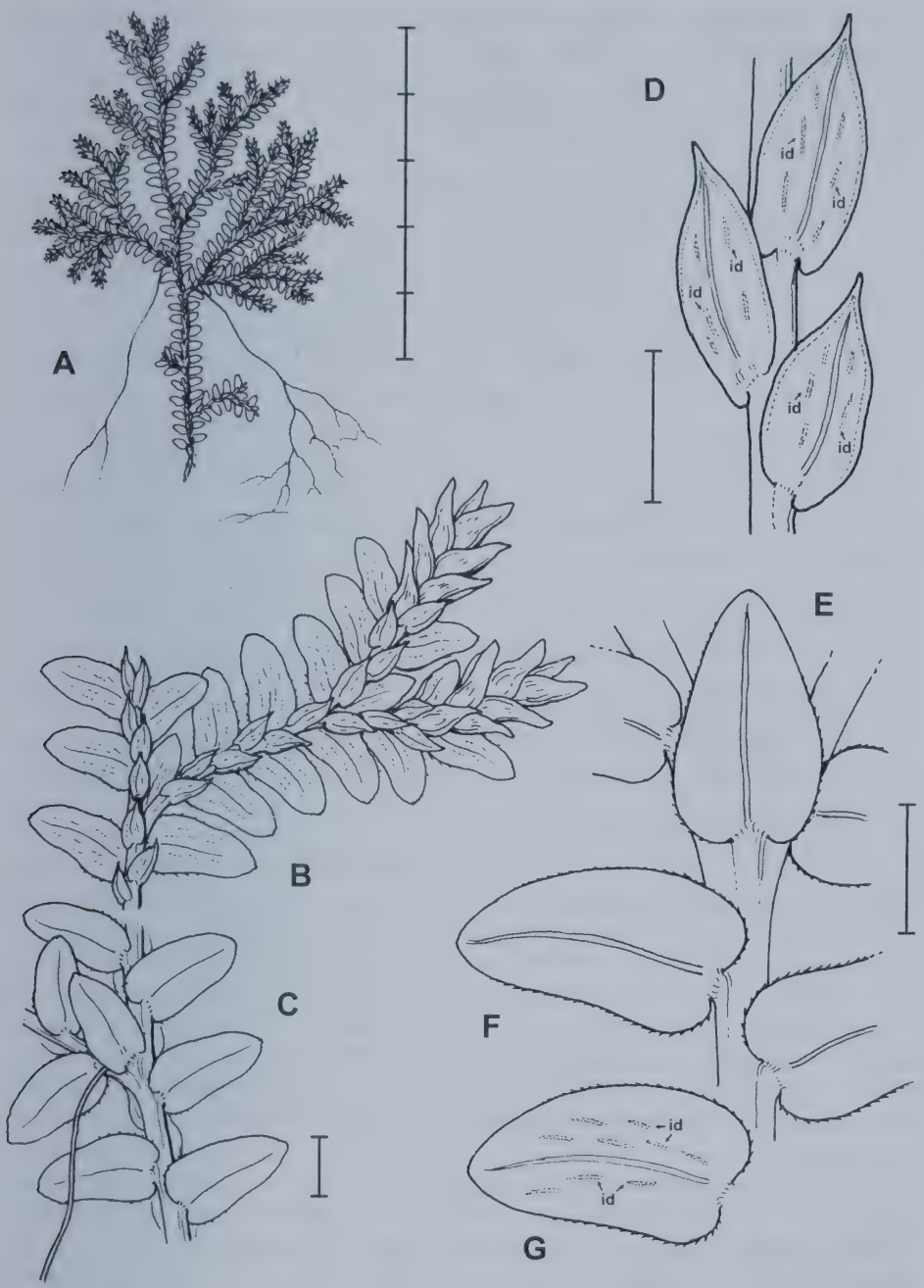

FIG. 1. A–F. *Selaginella hyalogramma* sp. nov. (*Davidse & González 21525*, holotype, MO). A. Habit. B. Detail of the upper surface of the stem, including strobili. C. Detail of the lower surface of the stem. D. Close-up of the upper surfaces of the median leaves; note idioblasts (id). E. Close-up of the lower surface of the axillary leaf. F. Close-up of the lower surface of lateral leaves. G. Close-up of the lower surface of a lateral leaf; note idioblast (id). (Scale bar: A = 5 mm; B & C = 1 mm; D–G = 1 mm).

serrulate apically, the basiscopic margins weakly hyaline, the cells as in acroscopic margins or pale green, serrulate; apices obtuse, dentate at tip; the upper surfaces with short, teeth-like hairs and stomata along basiscopic margins, the lower surfaces with stomata in 2–4 rows along the midribs. *Median leaves* distant, ascending, ovate to ovate-lanceolate, 1.0–1.7 × 0.5–0.7 mm; bases asymmetric or oblique; margins hyaline, in a band 1–3 cells wide, the cells elongate, straight-walled and papillate, parallel to margins, papillae in 1 row over each cell lumen and interconnected; margins serrate to serrulate; apices acuminate, the acumen ⅛ or less the length of the lamina, each acumen 0.1 to 0.2 mm long, dentate at tip and variously tipped by 1–3 teeth; the upper surfaces with stomata in 2–4 rows along the midribs and submarginal to marginal on inner margins, the lower surfaces without stomata. *Axillary leaves* similar to lateral leaves or more cordiform. *Strobili* resupinate, terminal on branch tips and main stem apices, lax, flattened, and dorsiventral, 4–11 mm long. *Sporophylls* dimorphic; *dorsal sporophylls* spreading, basally attached to the axis, with an adaxial laminar flap extending along ½–¾ the sporophyll length, each with a strongly developed and dentate keel along midribs; asymmetric to lanceolate, 1.6–2.1 × 0.5–0.9 mm; the bases rounded; the margins hyaline as in median leaves, serrate to serrulate; the apices acute; upper surfaces green with cells as in median leaves, including idioblasts and stomata, except for the half that overlaps the ventral sporophylls where the surfaces are hyaline with elongate, sinuate-walled cells, the lower surfaces silvery to silvery green, comprising elongate, sinuate-walled cells and with idioblasts; *ventral sporophylls* ascending, pseudopeltately attached to the axis, without an adaxial flap, each with a well-developed and slightly denticulate keel along midribs; ovate to broadly ovate, 1.4–1.85 × 0.6–0.72 mm; the bases rounded; the margins hyaline as in median leaves, serrate; the apices long- to short-acuminate, both surfaces almost colorless to completely hyaline, comprising elongate, sinuate-walled and glabrous cells, with conspicuous idioblasts and without conspicuous stomata. *Megasporangia* in two ventral rows; *megaspores* lemon yellow, with a prominent equatorial flange, the proximal faces reticulate with close reticula of low ridges and echinulate to papillate and foveolate microstructure, the distal faces reticulate with open reticula of high ridges and papillate and indistinct foveolate microstructure, 270–300 μm diam. *Microsporangia* usually in two dorsal rows; *microspores* orange, the proximal faces not studied in detail, the distal faces smooth, not measured.

Habitat and distribution.—*Selaginella hyalogramma* grows on mossy sandstone, on steep forested cliffs with open landslide areas at 430–530 m. It is only known from the type collection site in Venezuela.

Etymology.—The species epithet is derived from the Greek, *hyalinos*, glassy, transparent, and *gramme*, line; together these refer to the hyaline idioblasts on the upper and lower surfaces of the leaves and sporophylls.

Conservation status.—*Selaginella hyalogramma* is only known from a single collection made in Cerro La Camiri at El Tamá National Park in Táchira, Venezuela and, thus, at present, it is difficult to assign to it a definitive

conservation status. Nevertheless, judging by satellite images of the park, it seems moderately deforested at low to mid elevations and given the fact that this new species is known from only one location/population, a tentative Critically Endangered (CR) category according to IUCN (2012) is assigned to it.

Discussion

Selaginella hyalogramma is an epipetric species characterized by its ascending to suberect, non-articulate, not flagelliform, and not stoloniferous stems, axillary and filiform rhizophores, heteromorphic vegetative leaves with idioblasts on their upper and lower surfaces, dorsiventral and resupinate strobili with dimorphic sporophylls that also have idioblasts on their surfaces, dorsal sporophylls with a laminar flap, and megaspores with an equatorial flange. In addition, *S. hyalogramma* typically has stomata along the midribs and inner submarginal to marginal region on the upper surfaces of the median leaves (Fig. 2A & B), as well as along the basiscopic margins of the lateral leaves (Fig. 2F) and submarginal and marginal short or tooth-like hairs/trichomes on the upper surfaces near the basiscopic margins of the lateral leaves (Fig. 2F).

Interestingly, leaf marginal stomata are a more widespread character than previously realized in morphologically distinct *Selaginella* species from diverse regions of the world (Valdespino, 2015d). Furthermore, contrary to Youguang and Tan (2013) they ought to be considered functional (Valdespino, 2015d). Similarly, the occurrence of short or tooth-like hairs/trichomes seems to be another feature more prevalent in *Selaginella* species than hitherto acknowledged, particularly when detailed leaf surface SEM studies are conducted. This character is known to occur in other Neotropical species that belong in subg. *Heterostachys* sensu Jermy such as *S. correae* Valdespino, *S. minima* Baker, *S. pellucidopunctata* Valdespino, *S. popayanensis* Hieron., and *S. porphyrospora* Baker (Valdespino, 1993, 1995, 2015d), as well as in several very different and morphologically defined groups in subg. *Stachygynandrum* sensu Jermy. Among the latter, it is present, for example, in the *S. deltoides* group (Valdespino, 2016) and likewise in *S. potaroensis* Jenman and *S. vestiens* Baker (Valdespino, 2015d). In all mentioned taxa, short or tooth-like leaf surface hairs/trichomes occur variably throughout, submedially, submarginally, marginally, and apically on basiscopic halves of the laminae. The occurrence, diversity, and functions of leaf surface structures in plants have been amply discussed. Barthlott *et al.* (2017) extensively elaborated on them and concluded they are associated with evolutionary process over billions of years, whereas Kessler *et al.* (2007) stressed selected traits and hypotheses linked to leaf morphological characters. In both cited works, the potential roles of leaf hairs/trichomes and other types of leaf surface structures were invariably linked to environmental, physiological, and survival mechanisms. Recently, Wang *et al.* (2015), while examining leaf epidermal character variation and evolution in tribe Gaultherieae (Ericaceae), stressed the role of trichomes as barriers that protect plants from herbivores, UV radiation, and

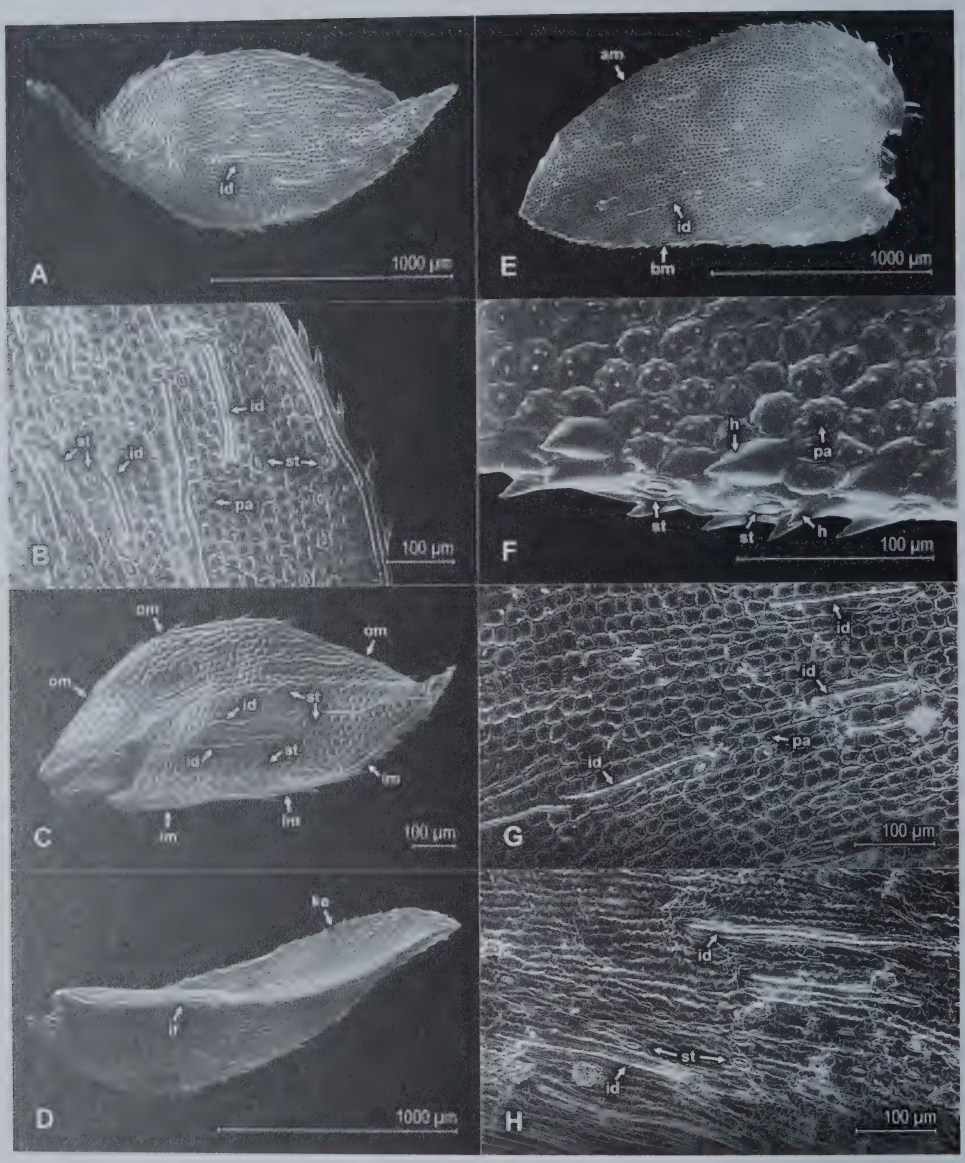

FIG. 2. A–H. *Selaginella hyalogramma* sp. nov. (*Davidse & González 21525*, holotype, MO). A. Upper surface of a median leaf, note idioblasts (id). B. Detail of upper surface of the same leaf as in A; note single papilla on a rounded cell (pa), medial and submarginal to marginal stomata (st), and single or paired idioblasts (id) with interconnected papillae. C. Upper surface of median leaf; note outer (om) and inner (im) margins, idioblasts (id), and stomata (st). D. Dorsal sporophyll; note laminar flap (lf) and keel (ke). E. Upper surface of lateral leaf; note acroscopic (am) and basiscopic (bm) margins, and idioblasts (id). F. Detail of basiscopic margin of the same leaf as in E showing very short, submarginal to marginal hairs (h), marginal stomata (st), and papillae (pa) on rounded cells. G. Detail of upper surface of lateral leaf; note rounded to polygonal, sinuate-walled cells, papillae (pa), and idioblasts (id). H. Detail of lower surface of lateral leaf; note elongate, sinuate-walled cells, stomata along midrib region, and idioblasts (id).

excessive transpiration. Similarly, in *Selaginella*, leaf surface hairs/trichomes might also play such roles, particularly those associated with: a) defense against herbivory, b) water and nutrient absorption, c) humidity retention and, thereby, reduction of transpiration rate, and d) redirection and absorption of light wavelength energy, which alone or jointly with the previous examples act during photosynthesis. Nevertheless, to my knowledge, the functionality of leaf surface hairs/trichomes on *Selaginella* has yet to be experimentally explored.

Selaginella hyalogramma appears morphologically close to and can be confused with *S. muscosa* Spring because both species have idioblasts on the upper and lower surfaces of the leaves and sporophylls, lateral leaves that are similar in shape and size, and yellow megaspores. It is also morphologically similar to *S. pellucidopunctata*, which has idioblasts on the lower surfaces of the lateral leaves and in both surfaces of the ventral sporophylls, in addition to yellow megaspores. Furthermore, *S. hyalogramma* and *S. pellucidopunctata* likewise share ascending to suberect stems, while these are creeping in *S. muscosa*. *Selaginella hyalogramma* differs from *S. muscosa* by having median leaves that are lanceolate (vs. broadly ovate or ovate-lanceolate) with asymmetric or oblique (vs. asymmetric or cordate to semicordate or rounded) bases, acuminate (vs. long-aristate) apices, each ⅛ or less (vs. ¼–½) the length of the lamina, the lateral leaf apices obtuse (vs. acute to shortly acuminate), the megaspore proximal faces reticulate with close reticula of low ridges (vs. seemingly smooth to granular or striate marginally), and the distal faces of the microspores smooth (vs. clavate). Recently, Bauer *et al.* (2016) reported the proximal faces of *S. muscosa* megaspores as having a less prominent equatorial flange and the overall surface sculpturing pattern more striate, as well as the distal faces reticulate, with the reticula composed of low muri. Nevertheless, as depicted here (Fig. 3E & G) and in Valdespino (1995), the proximal faces of *S. muscosa* megaspores have a very prominent and often perforate equatorial flange, and a seemingly smooth to granular surface sculpturing pattern, as well as a marginally striate ornamentation, while the distal faces are reticulate with high muri. These variations could be explained as proposed in Valdespino (1995) by: a) intraspecific variation and b) incipient speciation in *S. muscosa* or c) the degree of megaspore maturity used in these studies, d) the methodology employed for SEM studies and e) even possible misdetermination of examined specimens. Whichever the case, megaspore-sculpturing patterns of *S. hyalogramma*, as depicted in this paper, are quite different from those of *S. muscosa* as shown here, as well as in Valdespino (1995) and Bauer *et al.* (2016).

Selaginella hyalogramma can be separated from *S. pellucidopuntata* by the upper surfaces of the leaves comprising quadrangular or rounded cells, many of which are covered by 1–6 (vs. 7–30) papillae and with (vs. without) conspicuous idioblasts, the median leaf apices acuminate (vs. long-aristate), each acumen ⅛ or less (vs. each arista ½ or more) the length of the lamina, 0.1 to 0.2 (vs. 0.5–0.7) mm long, the lateral leaves with obtuse (vs. acute to short-

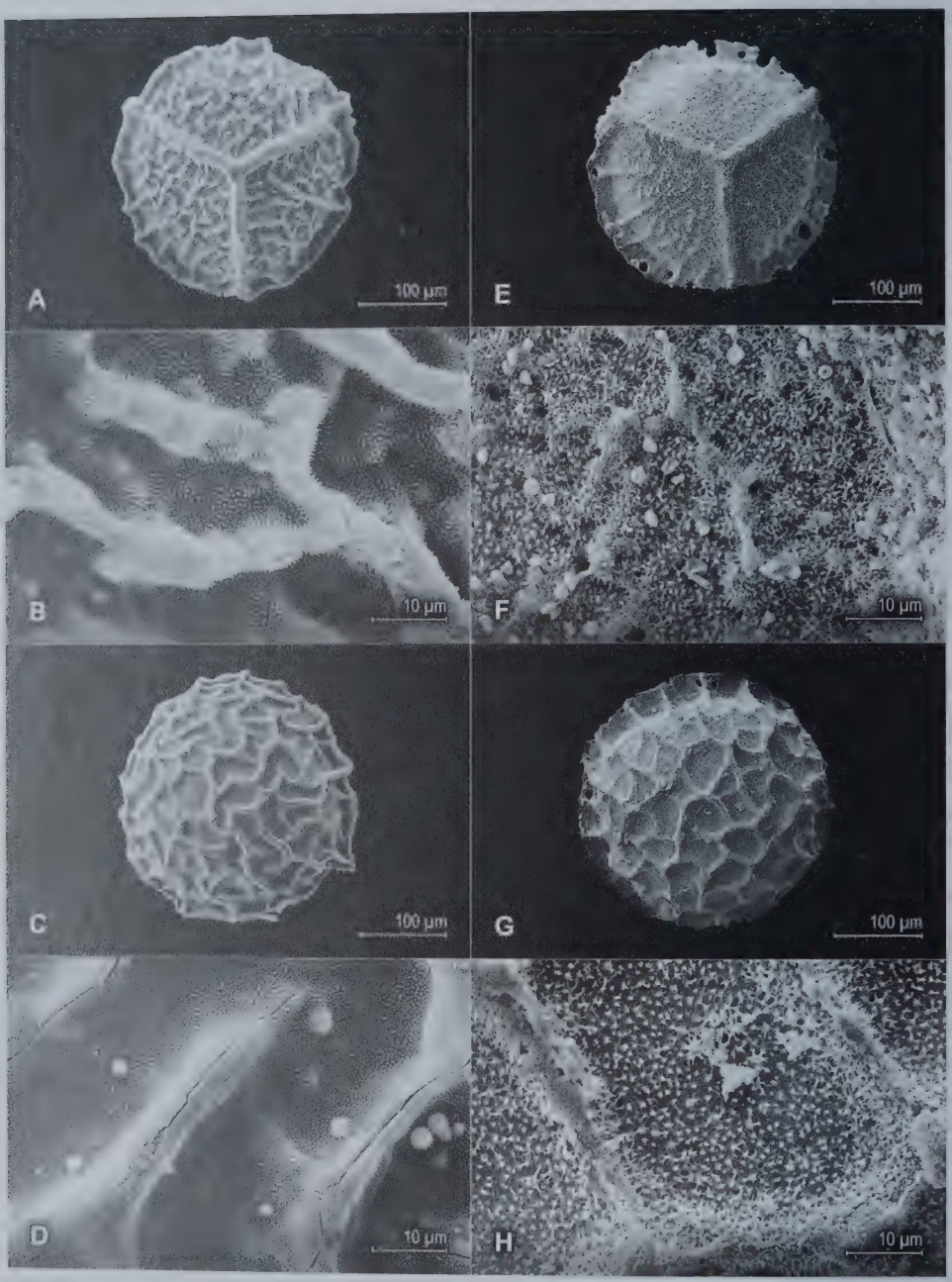

FIG. 3. SEM micrographs of *Selaginella* megaspores. A–D. *S. hyalogramma* sp. nov. (*Davidse & González 21525*, holotype, MO). A. Megaspore proximal face; note equatorial flange and reticulate sculpturing pattern. B. Close-up of megaspore proximal face. C. Megaspore distal face. D. Close-up of megaspore distal face. E–H. *S. muscosa* (*Rose & Russell 20441*, NY). E. Megaspore proximal face; note equatorial flange with perforations. F. Close-up of megaspore proximal face. G. Megaspore distal face. H. Close-up of megaspore distal face.

acuminate) apices, and the distal faces of the microspores smooth (vs. capitate).

The smooth distal faces of the microspores of *Selaginella hyalogramma* may relate it to *S. gynostachya* Valdespino, *S. karowtipuensis* Valdespino, and *S. seemannii* Hieron. (Valdespino, 1995). Additionally, *S. hyalogramma* and *S. seemannii* both have ascending to suberect stems, while in *S. gynostachya* and *S. karowtipuensis* stems are creeping. *Selaginella hyalogramma* can be separated from *S. gynostachya* and *S. karowtipuensis* by its asymmetric or oblique median leaf bases (vs. subtruncate to rounded in *S. gynostachya* and subcordate in *S. karowtipuensis*) and megaspores 270–300 μm in diam. (vs. 320–340 μm in *S. gynostachya* and 300–320 μm in *S. karowtipuensis*). It additionally differs from *S. gynostachya* by its ovate to ovate-lanceolate (vs. elliptic to ovate-elliptic or broadly ovate) median leaves with the lamina 1.0–1.7 mm (vs. 1.7–2.3 mm) wide, and broadly ovate to broadly ovate-deltate (vs. oblong to oblong-ovate) lateral leaves. *Selaginella hyalogramma* is easily set aside from *S. seemannii* by its conspicuous (vs. absent) idioblasts on the upper surfaces of the leaves, broadly ovate to broadly ovate-deltate (vs. ovate-oblong to ovate-lanceolate) lateral leaves, and median leaves 1.0–1.7 × 0.5–0.7 mm (vs. [1.25–] 1.6–2.85 × [0.7–] 1.0–1.75) mm.

Key to *Selaginella hyalogramma* and Morphologically Close Species

1. Stems ascending to suberect.
   2. Upper surfaces of leaves and of dorsal sporophylls with conspicuous idioblasts; lateral leaf apices obtuse; median leaves lanceolate with acuminate apices, each acumen ⅛ or less the length of the lamina . . . . . *S. hyalogramma*
   2. Upper surfaces of leaves and of dorsal sporophylls without idioblasts; lateral leaf apices broadly acute or acute to shortly acuminate; median leaves elliptic to ovate-lanceolate or ovate-elliptic with acuminate or long-aristate apices, each acumen or arista ¼–½ or more the length of the lamina.
      3. Median leaf apices long-aristate, each arista ½ or more the length of the lamina; acroscopic margins of the lateral leaves ciliate along proximal ½, otherwise dentate distally; upper surfaces of dorsal sporophylls with conspicuous stomata throughout, the upper and lower surfaces of ventral sporophylls with idioblasts; rhizophores filiform, each 0.1 to 0.2 mm diam . . . . . *S. pellucidopunctata*
      3. Median leaf apices acuminate, each acumen ¼ the length of the lamina; acroscopic margins of the lateral leaves serrate throughout or only along proximal ½ and on distal ¼; upper surfaces of dorsal sporophylls with stomata only along midribs, the upper and lower surfaces of ventral sporophylls without idioblasts; rhizophores stout, each 0.25–0.65 mm diam . . . . . *S. seemannii*
1. Stems creeping.
      4. Median leaf bases subcordate, the inner bases rounded and the outer bases distinctly auricled with the auricles free from the stems; rhizophores stout, each 0.5–0.88 mm diam.; lateral leaves 3.0–5.3 mm long . . . . . *S. karowtipuensis*
      4. Median leaf bases asymmetric, the inner bases truncate and the outer bases weakly auricled, or subcordate to rounded, if weakly developed outer auricles present, these adnate to the stems; rhizophores filiform or stout, each 0.1–0.65 mm diam.; lateral leaves 2.0–4.0 mm long.
         5. Lateral leaf upper surfaces without idioblasts, the lower surfaces frequently with conspicuous or indistinct idioblasts; median leaves

elliptic to ovate-elliptic, each 1.7–2.3 mm long with acute to shortly acuminate apices, the acumen, if present, less than ⅛ the length of the lamina, each 0.1–0.26 mm long mm; rhizophores stout, each (0.28–)0.34–0.65 mm diam ..... *S. gynostachya*

5. Lateral leaf upper and lower surfaces frequently with idioblasts or with idioblasts present only on lower surfaces; median leaves broadly ovate or ovate-lanceolate, each (0.86)1.0–1.6 mm long with short to long-acuminate or long-aristate apices, the acumen or arista ¼–½ or more the length of the lamina, each (0.32–)0.5–0.9 mm long; rhizophores filiform, each 0.1 to 0.2 mm diam ..... *S. muscosa*

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Four New Species of *Adiantum* (Pteridaceae) from the Guianas

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Abstract.—We describe, discuss, and illustrate four new species of the fern genus *Adiantum* (Pteridaceae): one from Guyana (*A. cremersii* Boudrie & J. Prado) and two from French Guiana (*A. granvilleanum* Boudrie & J. Prado and *A. latipinnulum* Boudrie & J. Prado), and *A. rivularis* Boudrie & J. Prado that occurs in Guyana and French Guiana. The typification and application of the name *A. oyapokense* Jenman are also discussed.

Key Words.—Ferns, French Guiana, Guyana, South America, taxonomy

Adiantum L. (Pteridaceae) is a monophyletic genus (Rothfels and Schuettpelz, 2014; Rothfels *et al.*, 2015; Pryer *et al.*, 2016) and easily recognized by the sporangia borne on the false indusium itself. It is a group with more than 225 species and widely distributed in the tropics (PPG I, 2016).

Although *Adiantum* is a very distinct genus, the taxonomy of some species or group of species is a challenge, because *Adiantum* is a large genus with a broad distribution. Several new species have been recognized recently (e.g., in the last 10 years) especially from the Americas: From USA, 1 sp. (Huiet *et al.*, 2015); Mexico, 1 sp. (Hirai *et al.*, 2014); Costa Rica and Nicaragua, 2 spp. each (Rojas, 2007, 2008; Rojas and Martínez, 2012); Cuba, 1 sp. (Caluff, 2009); French Guiana, 1 sp. (Zimmer, 2007); Ecuador, 1 sp. (McCarthy and Hickey, 2011); Brazil, 1 sp. (Prado and Hirai, 2013); Argentina, 1 sp. (Sundue *et al.*, 2010).

Cremers (1997) reported 18 spp. of *Adiantum* from the region of Central French Guiana. In the region of the three Guianas 32 species of *Adiantum* occur (Guyana with 25 species, Suriname with 23 spp., and French Guiana with 27 spp.; M. Boudrie & G. Cremers, updated personal checklist as of March 2017).

During the revision of the fern genus *Adiantum* for the Flora of the Guianas, the morphology and distribution of some specimens aroused our attention; these were revealed to be additional undescribed species in the genus. The main objectives of this paper are to describe these new taxa and to discuss the most morphologically similar species.

Material and Methods

Morphological features such as rhizome, lamina division, shape of the pinnules, and indument were the main tools used to recognize the new taxa. The voucher specimens used in this study are deposited in the following herbaria: CAY, NY, P, SP, U, and UC.

Additional information, enclosed in brackets, related to localities, geographic coordinates, and elevation, was added and estimated by us.

Results and Discussion

Based on morphology and geographical distribution, the four new species of *Adiantum* we discovered in the Guianas are presented. One species, *Adiantum cremersii*, is known only from Guyana, District of Rupununi, and two other species, *A. granvilleanum* and *A. latipinnulum*, occur only in French Guiana. *Adiantum rivularis*, known from French Guiana and Guyana, shows the most remarkable habit of growing pendent on the banks of rivers (Fig. 1A–E).

New Species:

Adiantum cremersii Boudrie & J. Prado, ***sp. nov.*** **Type.**–Guyana. Rupununi Distr.: Kanuku Mts., Crabwood Cr., Camp 2, 03°07′N, 59°06′W, elev. 260 m, 31 Jan 1994, *M.J. Jansen-Jacobs, B.J.H. ter Welle, A. Chanderbali, U. Raghoenandan, V. James 3486* (holotype: CAY barcode CAY043931!; isotypes: U barcode U0238283!, UC accession UC1612347!). **Figs. 2A–E, 3**

Adiantum cremersii *is a distinct species by having a long-creeping rhizome; fronds 2-pinnate; petiole and rachises with two kinds of scales (filiform and arachnidoid); pinnules 4–10 times longer than wide, basiscopically slightly excavate, abaxially with scales sparse, filiform (2-cells wide at base), reddish-brown; and indusia pubescent, the hairs reddish-brown, ca. 0.2 mm long.*

Rhizomes horizontal, long-creeping, with fronds 1–1.5 cm apart, 3 mm thick, scaly, the scales brown to dark brown, lanceolate to long-triangular. *Fronds* 65 cm long; *petioles* up to 40 cm long, blackish, lustrous, with two kinds of scales, the scales filiform with pectinate bases and arachnidoid scales (hair-like), both sparse and appressed; *laminae* 30–35 × 25–30 cm, widely triangular, not reduced at base, herbaceous, 2-pinnate, with two pairs of opposite to alternate pinnae and a terminal conform one; adaxial face of lamina glabrous, with idioblasts easily visible between veins, and abaxial face with scales sparse, filiform (2-celled at base), reddish-brown, 0.5–1 mm long, mainly on the main veins and mostly on the basiscopic side; *rachises* scaly, the scales of two kinds (one filiform with pectinate base, 0.5–1.2 mm long, with 4–8 celled wide at base and the second arachnidoid like those of the petioles, ca 0.5 mm wide); *pinnae* 12–18 × 5–6

FIG. 1. A–E. *Adiantum rivularis*. A. (Saut Dalles, Mana Riv., 2013). B. (Ilet Lézard, Mana Riv., 2013). C. (Ilet Lézard, Mana Riv., 2013). D. (Saut Tamanoir, Mana Riv., 2013). E. (Saut Belle Etoile, Mana Riv., 2013). All photos by V. Pelletier.

cm, obovate-oblanceolate, reduced at base with 0.6 cm diameter rounded pinnules, with 15–19 pinnules per pinna, apex slightly reduced to a small group of 1 cm long pinnules, and a long terminal pinnule, narrowly subrhombic to lanceolate, 3–4 cm long; *pinnules* 2–4 × 0.5–0.7 cm, 4–10 times longer than wide, sessile to short-petiolulate, dimidiate, basiscopic side

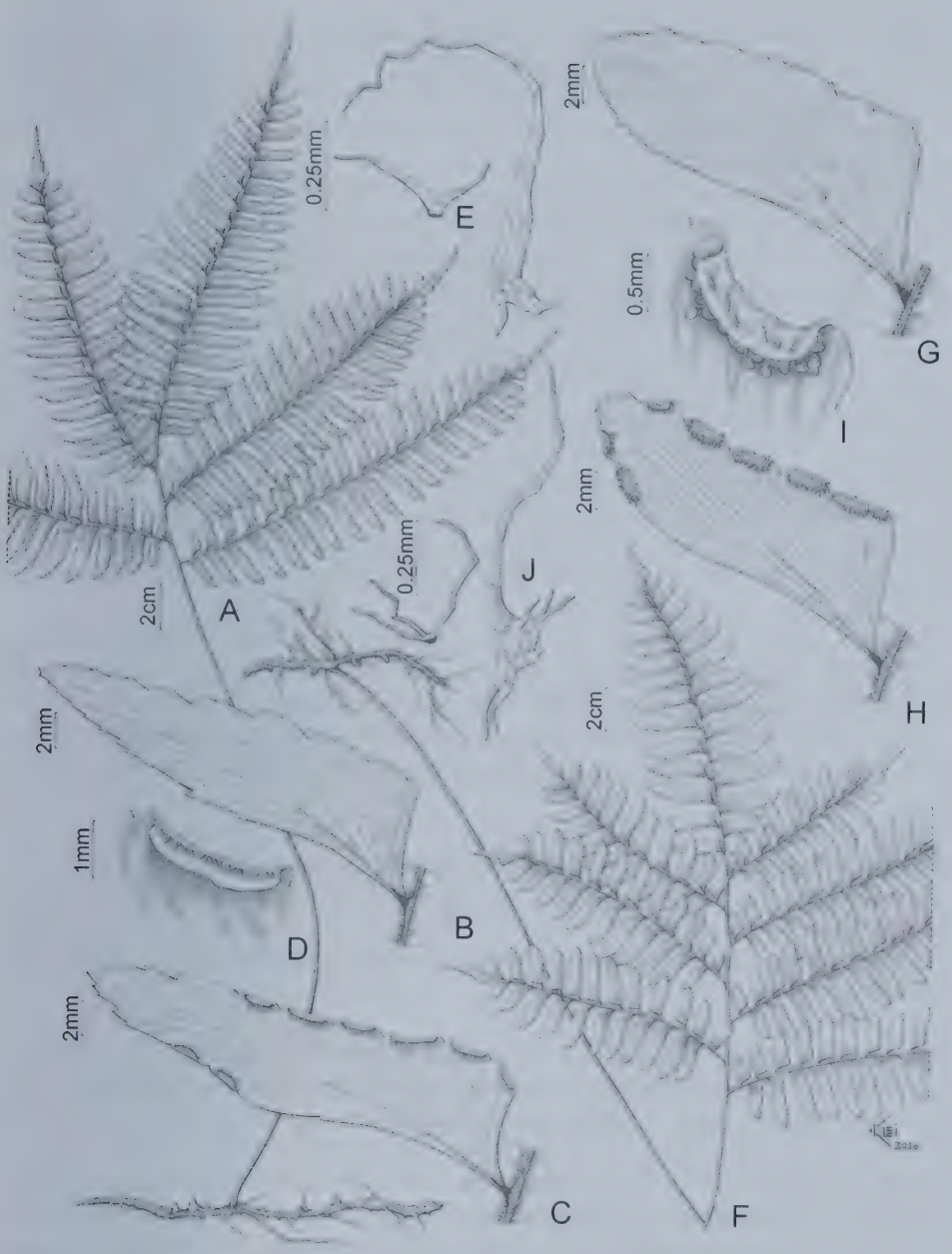

FIG. 2. A–E. *Adiantum cremersii*. A. Habit. B. Abaxial view of the sterile pinnule and a detail of its margin. C. Abaxial view of fertile pinnules. D. Detail of the indusium. E. Scales from rachises (A–E, from *Jansen-Jacobs 3486*, CAY). F–J. *A. granvilleanum*. F. Habit. G. Abaxial view of the sterile pinnule and a detail of its margin. H. Abaxial view of fertile pinnules. I. Detail of the indusium. J. Scales from rachises (F–J, from *de Granville 4668*, CAY).

slightly excavate and acroscopic side straight, pointed at apex, margins double serrate. *Sori* discontinuous, mainly on the acroscopic part of the pinnules, only a few on the basiscopic side; *indusia* narrow and elongated, 2–2.5 mm long, blackish, hairy, the hairs reddish-brown, ca. 0.2 mm long; spores not seen.

Distribution and ecology.—To our present knowledge, only known from the type from Guyana. Inside forest, growing on brown loamy sand; ca. 260 m elev.

Etymology.—The specific epithet honours our friend Dr. Georges Cremers, specialist of the pteridophytes of the Guianas.

This species resembles *Adiantum tetraphyllum* Humb. & Bonpl. ex Willd. (which also occurs in the same geographical area), in its long-creeping rhizomes, with distant fronds, and terminal pinnules narrowly sub-rhombic to lanceolate. However, *A. cremersii* differs in the shape of the lateral pinnules (which are 4–10 times longer than wide) and base of the pinnules being slightly excavate basiscopically (vs. pinnules 2–4 times longer than wide and base straight basiscopically in *A. tetraphyllum*). Additionally, *A. cremersii* has filiform and arachnidoid scales on the petioles and rachises (vs. filiform and lanceolate scales in *A. tetraphyllum*). These scales are most easily observed on young fronds.

Spores of this new species were not observed because the type is an old plant. Only fragments of empty sporangia can be seen and the sporangia were well-formed, not suggesting a specimen of hybrid origin.

Adiantum cremesii came from a place well-explored in Guyana (Rupununi Distr.: Kanuku Mts.). It seems to be a rare species, as it was collected only one time.

Adiantum granvilleanum Boudrie & J. Prado, ***sp. nov.*** **Type.**–French Guiana. Gros Saut, fleuve Mana, rive droite, emplacement d'un ancien village, 4°40′28″N, 53°37′18″W, 26 juillet 1981, *J.-J. de Granville 4668* (holotype on 3 sheets: CAY barcodes CAY088869!, CAY088870!, CAY155844!). **Figs. 2F–J, 3**

Adiantum granvilleanum *can be recognized by its long-creeping rhizome; laminae herbaceous, 2-pinnate; petioles and rachises covered with two kinds of scales: filiform scales with pectinate base and arachnidoid scales; indusia pubescent to glabrous; the hairs brown, 0.1–0.2 mm long.*

Rhizomes horizontal, long-creeping, with fronds 2–4 cm apart, 3 mm thick, scaly, the scales entire, lanceolate to triangular, brown, 1.5–2 mm long. *Fronds* 60–70 cm long; *petioles* 35–40 cm long (1/2 frond length), black and lustrous, with two kinds of scales (filiform scales with bases pectinate and hair-like arachnidoid scales), both sparse and appressed; *laminae* 25–30 × 23–27 cm, widely oblong, not reduced at base, herbaceous, 2-pinnate, with 3 or 4 pairs of alternate pinnae and a 14–17 × 4.5–6 cm terminal conform one; adaxial face of lamina glabrous and abaxial face slightly glaucous and almost glabrous, only with sparse filiform scales with pectinate base on the veins; *rachises and costae* with abundant appressed arachnidoid scales mixed with filiform scales

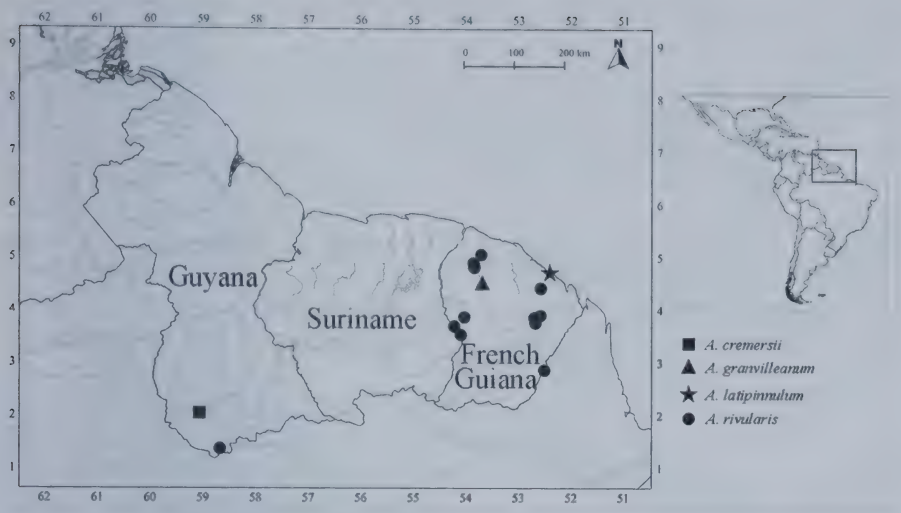

FIG. 3. Distribution of *Adiantum cremersii*, *A. granvilleanum*, *A. latipinnulum*, and *A. rivularis*.

0.8–2 mm long, with pectinate bases; *pinnae* 10–13 × 4–5 cm, oblanceolate, slightly reduced at base, with (10–)15–18 pinnules per pinna, apex pinnatifid with a conform terminal sub-rhombic pinnule; *pinnules* 2–3 × 0.5–0.8 cm, 3–5 times longer than wide, sessile to short petiolulate, dimidiate, slightly falcate, with round to acute apex, margins flat and finely denticulate. *Sori* discontinuous, mainly of the acroscopic part of the pinnules, only a few ones on the basiscopic side; *indusia* narrow and elongated, 2–3 × 0.4 mm, black, pubescent to glabrous, the hairs brown, 0.1–0.2 mm long; spores not seen.

Distribution and ecology.—To our present knowledge, only known from the type from French Guiana. Growing along margin of a trail; secondary forest; elevation 40 m.

Etymology.—The specific epithet honors Jean-Jacques de Granville, a French botanist of the Institut de Recherche pour le Développement (IRD) in French Guiana, who is a specialist in palm systematics and an extraordinary collector of ferns in the Guianas.

Adiantum serratodentatum Humb. & Bonpl. ex Willd. is the most similar species in having rhizomes long-creeping; fronds distant, laminae 2-pinnate, and petioles and rachises with arachnidoid and filiform scales. However, *A. granvilleanum* can be distinguished from *A. serratodentatum* by its herbaceous laminae (vs. coriaceous in *serratodentatum*); median pinnules 3–5 times longer than wide and margins flat (vs. 1–2 times longer than wide and margins strongly revolute).

Spores of this species were not observed. The fertile frond of the holotype has sporangia well-formed and not indicating to be a hybrid. The sporangia are completely opened and without spores.

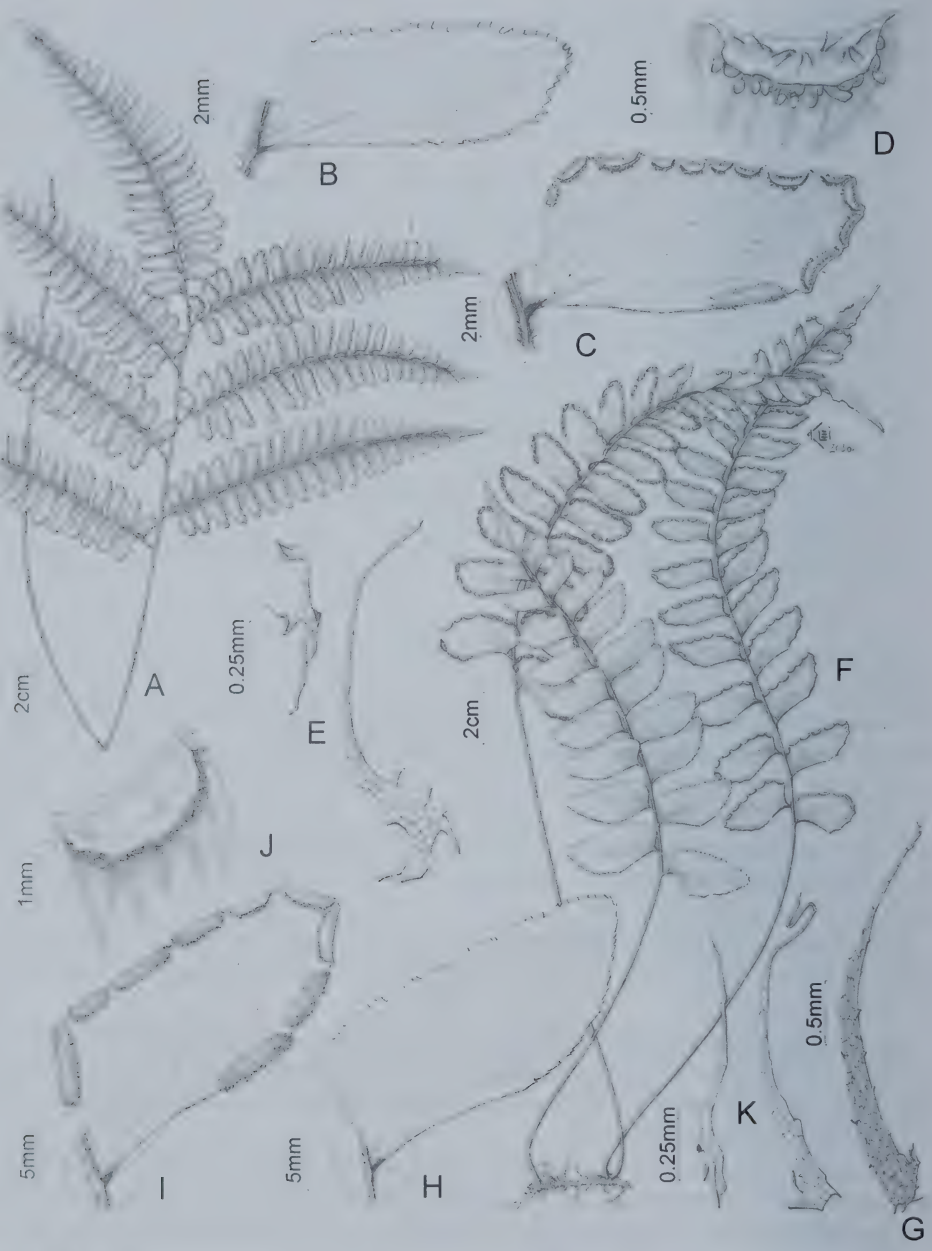

FIG. 4. A–E. *Adiantum latipinnulum*. A. Habit. B. Abaxial view of the sterile pinnule and a detail of its margin. C. Abaxial view of fertile pinnules. D. Detail of the indusium. E. Scales from rachises (A–E. from *de Granville 2833*, CAY). F–K. *A. rivularis*. F. Habit. G. Rhizome scale. H. Abaxial view of the sterile pinnule and a detail of its margin. I. Abaxial view of fertile pinnules. J. Detail of the indusium. K. Scales from rachises. (F–K, from *Boudrie 3902*, CAY).

Adiantum granvilleanum was also collected one time, during an exploration mission carried out 35 years ago along the Mana river by the botanists of ORSTOM, and notably J.-J. de Granville who was exercised to ferns. Since 1981, there has been no other botanical mission in the area with experienced botanists. This explains the fact that this collection is unique. Up to now, there is no argument to say that this species is rare or not in the area.

Adiantum latipinnulum Boudrie & J. Prado, ***sp. nov.*** TYPE.–FRENCH GUIANA. Chemin Vidal, Rémire, 4°52′30″N, 52°17′20″W, 4 février 1978, *J.-J. de Granville 2833* (holotype: CAY barcode CAY088867!; isotypes: CAY088868!; P, not seen; Z barcode Z000108501!). **Figs. 3, 4A–E.**

Adiantum latipinnulum *is recognized by the laminae 2-pinnate; pinnules wide, 2–3* x *0.5–1.0 cm; petiole and rachises with sparse indument composed of filiform and arachnidoid scales; pinnules abaxially with filiform scales with pectinate bases, mainly on the veins and at pinnule bases; indusia with reddish-orange short hairs, ca. 0.1–0.2 mm long.*

Rhizomes long-creeping, with fronds 1–2 cm apart, scaly, the scales not seen. *Fronds* 75 cm long; *petioles* 40 cm long (1/2 frond length), black and lustrous, with two kinds of scales (sparse filiform scales, 2–3 mm long, pectinate at the base, and appressed hair-like arachnidoid scales); *laminae* 25–30 × 25–30 cm, widely oblong, not reduced at base, herbaceous, 2-pinnate, with 3 or 4 pairs of alternate pinnae and a conform terminal one, adaxial face of lamina glabrous and abaxial face with very sparse filiform scales, 0.4–0.6 mm long, with pectinate bases, on the veins; *rachises and costae* with two kinds of scales (arachnidoid scales plus filiform scales 0.8–2 mm long, both appressed like those of the petioles); *pinnae* 12–18 × 4–5 cm, oblanceolate, slightly reduced at base, with 15–18 pinnules per pinna, apex pinnatifid with a conform terminal sub-rhombic pinnule; *pinnules* 2–3 × 0.5–1.0 cm, 2 or 3 times longer than wide, short-petiolulate, dimidiate, the basal acroscopic pinnule rounded and 1–1.5 cm diam. *Sori* discontinuous, mainly on the acroscopic part of the pinnules, only a few around the apex of the pinnule; *indusia* narrow and elongated, 1.5–4 × 0.2–0.4 mm, brownish-orange, with lacerate margin and a few reddish-orange short hairs, ca. 0.1–0.2 mm long; spores trilete, tetrahedral-globose, the angles prolonged, light brown.

Distribution and ecology.—To our present knowledge, only known from the type from French Guiana. In degraded forest; altitude 25 m.

Etymology.—The specific epithet refers to the wide pinnules that resembles the pinnules of *Adiantum latifolium* Lam.

Adiantum latipinnulum is very similar in habit to *A. latifolium*, but can be distinguished by the presence of an indument of scales with pectinate bases on the pinnules abaxially and also by the hairs on the indusia. *Adiantum latifolium* has pinnules and indusia glabrous. Another similar species is *Adiantum windischii* J. Prado, which differs by the presence of hairs on the pinnule veins abaxially and the glabrous indusium.

Our confirmation of the type specimen as a new species, *Adiantum latipinnulum*, is very recent (2015). Although the area of collection is near Cayenne, in the coastal region of French Guiana, and has been visited several times by botanists, it has not been possible to find again the plant, the edges of the trail along which it was growing having been strongly modified since 1978 (secondary and degraded forest). This area will need further exploration. However, despite the number of fern collections, in the vicinity of Cayenne, this unique collection shows that it is a rare species.

Adiantum rivularis Boudrie & J. Prado, ***sp. nov.*** **Type.**–French Guiana. Bord de la crique Arataï, en amont de la station de l'Arataï, à 60 km au sud-ouest de Régina, 3°59′N, 52°35′W, alt. 20 m, 24 février 2003, *M. Boudrie & groupe FOG 3902* (holotype: CAY barcode CAY037782!, isotypes: P barcode P01454076!; UC accession UC1785002!). **Figs. 1A–E; 3, 4F–K.**

Adiantum rivularis *is easily recognized by its habit of pendent growth; laminae chartaceous, apex progressively reduced, and pinnae abaxially glaucous, with sparse filiform scales with pectinate base.*

Rhizomes long-creeping, nodose, with fronds 0.5 mm apart, 2 mm thick, covered with dense, tan, entire, appressed to slightly recurved scales, 2 mm long, narrowly lanceolate. *Fronds* up to 50–55 cm long, pendent; *petioles* 20–25 cm long, generally 1/2 the frond length, black, with very sparse, tan, filiform (1-celled wide), 2 mm long scales, slightly widened at base (2-celled), and with numerous appressed arachnidoid scales, more abundant at the top of the petiole; *laminae* 20–25 × 7–9 cm, oblong, not reduced at base, pinnate, chartaceous, with 10–15 pairs of alternate pinnae and progressively reduced at apex; adaxial face of lamina glabrous, with idioblasts visible between veins, and abaxial face glaucous and with sparse scales, mostly on the veins at pinna base, the scales filiform with pectinate bases; *rachises* black to dark brownish, with numerous appressed arachnidoid scales mixed with filiform, tan, 2 mm long scales, with pectinate bases; *pinnae* 4–5 × 1–2 cm, dimidiate, stalked to sessile, the stalk 3–5 mm long, oblong, excavate basiscopically, with apex generally rounded (sometimes acute); proximal pinnae sometimes deltoid and asymmetrical, or occasionally pinnate, with 1 or 2 pinnules. *Sori* discontinuous, all around the pinnules except on the basiscopic excavate side; *indusia* narrow and elongated, erose, 3–4 × 0.5 mm, black-brownish, with irregular, light brown to russet margins, glabrous; spores trilete, tetrahedral-globose, light brown.

Distribution and ecology.—An overlooked species, apparently frequent along rivers in French Guiana, Guyana, and probably present in Brazil (Amapá, Pará), Suriname, and Venezuela. On vertical lateritic or argillaceous banks, near river margins; 5–20 m elev.

Etymology.—The specific epithet refers to the preferred habitat of the species: on banks near river margins.

PARATYPES.—FRENCH GUIANA. Talus ombragé de bord de rivière, vallée du Grand Inini, abords du camp d'écotourisme de Saut Sonnelle, à 4,5 km à l'est-nord-est de Maripasoula, 03°59′N, 53°58′W, alt. 100 m, 27 nov 2003, *M. Boudrie 3955* (CAY); Paroi verticale, berges du Marouini, près de la borne géodésique Marouini, rivière Marouini, commune de Maripasoula, [03°38′N, 54°02′W],10 nov 1977, *G. Cremers 5006* (CAY, P, Z); Berges inondables, forêt sur schistes du Bonidoro, Saut Dalles, fleuve Mana, rive droite, à 65 km au sud-sud-est de Saint-Laurent-du-Maroni, 04°58′N, 53°47′W, alt. 90 m, 19 juil 1981, *J.-J. de Granville 4588* (BR, CAY, P, Z); Bord de la rivière, Saut Bief, rivière la Comté, Cacao, commune de Roura, 04°34′N, 52°28′W, alt. 10 m, 24 juin 1965, *R.A. Oldeman 1407* (CAY, P); Berges latéritiques abruptes de la rivière, Dégrad Cacao, vallée de la Comté, Cacao, commune de Roura, 04°34′N, 52°28′W, alt. 10 m, 8 déc 2013, *V. Pelletier 115* (CAY, P, SP); Sur les berges rocheuses abruptes de la rivière, Saut Dalles, vallée de la Mana, à 65 km au sud-sud-est de Saint-Laurent-du-Maroni, alt. 20 m, 04°58′N, 53°47′W, 27 juin 2013, *V. Pelletier 117* (B, BM, CAY, K, NY, P, SP, UC, US); Berges latéritiques abruptes de la rivière, crique Arouani, vallée de la Mana et affluents, à 65 km au sud-sud-est de Saint-Laurent-du-Maroni, alt. 90 m, 04°58′N, 53°47′W, 27 juin 2013, *V. Pelletier 118* (CAY, MO, P); Berges latéritiques abruptes de la rivière, Ilet Lézard, vallée de la Mana, à 65 km au sud-sud-est de Saint-Laurent-du-Maroni, alt. 35 m, 05°03′N, 53°48′W, 27 juin 2013, *V. Pelletier 119* (BM, CAY, NY, P, SP, UC); Berges latéritiques abruptes de la rivière, Saut Belle Etoile, vallée de la Mana, à 50 km au sud-sud-est de Mana, alt. 10 m, 05°13′N, 53°39′W, 27 juin 2013, *V. Pelletier 123* (BBS, CAY, SP, UC, US); Berges latéritiques abruptes de la rivière, Saut Moulouwa, vallée de l'Oyapock, à 20 km au sud de Camopi, alt. 70 m, 02°57′N, 52°23′W, 19 nov 2013, *V. Pelletier 127* (CAY, K, MO, P, SP); Berges latéritiques abruptes de la rivière, Saut Grand Machikou, vallée de l'Approuague, à 70 km au sud-ouest de Régina, alt. 30 m, 03°53′N, 52°34′W, 22 déc 2013, *V. Pelletier 177* (CAY); Berges latéritiques abruptes de la rivière, crique Ekini, vallée de l'Approuague, à 50 km au sud-ouest de Régina, alt. 16 m, 04°02′N, 52°28′W, 15 avril 2014, *V. Pelletier 197* (B, BBS, CAY, P, SP); Au confluent de la crique Arataïe, vallée de l'Approuague, à 60 km au sud-ouest de Régina, alt. 20 m, 03°58′N, 52°33′W, 22 déc 2013, *V. Pelletier 592* (CAY); Sur une butte longeant le fleuve, layon amont longeant le fleuve Lawa [= Maroni], Papaïchton, 03°48′N, 54°10′W, alt. 100 m, 30 août 1986, *C. Sastre, D. Bell & F. Crozier 8158* (CAY, P, US). GUYANA. U. Takutu-U, Essequibo, Acaraï Mts, Watuwau Creek near juncture with Chodikar River, 01°22′N, 58°42′W, alt. 250 m, 21 Feb 1994, *T.W. Henkel, M. Chin, R. Williams & R. James 4595* (CAY, US).

Adiantum rivularis is a remarkable species in its habit. It grows pendent on vertical banks, along river margins. *Adiantum petiolatum* Desv. is the most similar species but differs by the herbaceous laminae (vs. chartaceous in *A. rivularis*), terminated by a more or less conform, deltoid, pinna (vs. apex progressively reduced in *A. rivularis*) and pinnules abaxially glabrous (vs. with sparse filiform scales mostly on the veins in *A. rivularis*). Also, both species

differ in their habit, with *A. petiolatum* being erect while *A. rivularis* is pendent.

Lectotypification and new synonymy:

Adiantum* ×*oyapokense Jenman, Bull. Misc. Inform. Bot. Dept. Trinidad 19(App.): 85. 1899. LECTOTYPE (here designated).–FRENCH GUIANA. "In insulis sabulosis dumosis delapsi aricoto. Oyapoke superius, Guyane", Dec 1835, *F.M.R. Leprieur s.n.* (NY barcode NY00888038!; ISOLECTOTYPE: P barcode P01587464!).

Adiantum ×*variopinnatum* Jermy & T. Walker, Bull. Brit. Mus. (Nat. Hist.), Bot. 13(2): 254, f. 3. 1985. TYPE.–TRINIDAD. Tacarigua Ward, Tunapuna, two miles up Caura Road near river, ca. 60 m alt., on sides of gully in secondary forest, 6 Jul 1963, *A.C. Jermy & T.G. Walker J2070* (HOLOTYPE: BM barcode BM 000936715, photo!), ***syn. nov.***

After studying the type specimens of *Adiantum oyapokense* and *Adiantum* ×*variopinnatum* we concluded that they are conspecific and *A.* ×*oyapokense* is the oldest name for the hybrid *A. latifolium* × *A. petiolatum* that was described by Jermy and Walker (1985) as *A.* ×*variopinnatum*. The lectotype and isolectotype of *A.* ×*oyapokense* have abortive spores, showing the hybrid origin of the specimens.

Besides French Guiana, *Adiantum* ×*oyapokense* has been recorded in the following countries: Costa Rica, Nicaragua (Jermy 1995), and Trinidad. This hybrid occurs in low elevations between 15–60 m, on banks in secondary forest in loamy or clay soils.

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A Successful *in vitro* Propagation Technique for Resurrection Plants of the Selaginellaceae

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Abstract.—Resurrection plants of the Selaginellaceae are renowned for their ability to tolerate desiccation as well as the small size of their nuclear genomes. These traits position *Selaginella* as a promising model system to understand many aspects of plant evolution. However, there is not an established method for the laboratory cultivation of resurrection species of *Selaginella*. We explored methods of *in vitro* propagation for resurrection species of *Selaginella* and identified a set of successful techniques. Our *in vitro* propagation system included two main steps: surface-sterilized megaspores were cultured alone on C-Fern agar medium for three weeks, followed by the addition of surface-sterilized microspores to the germinated megaspore cultures for co-culture. Sporelings of *Selaginella eremophila* and *S. rupincola* were observed after 2–5 weeks of co-culture, and all sporelings survived. Our methods aim to further the interest and use of resurrection species of *Selaginella* for manipulative studies to better understand the biology of desiccation tolerance and their unique genome architecture.

Key Words.—*Selaginella*, microgametophytes, megagametophytes, fertilization, sporelings

Selaginella P. Beauv. (Selaginellaceae) is the largest genus of seed-free vascular plants with an estimated 700–800 species found on all continents except Antarctica (Jermy, 1956; Jermy, 1990; Zhou *et al.*, 2015). Radiating out of the humid tropics, many epiphytic and epipetric species in both tropical and arid environments have independently evolved varying degrees of desiccation tolerance. These adaptations are associated with significant ecological niche shifts from tropical climates into both the hot-arid environment of the desert and the cold-arid habitats of the alpine and tundra (Arrigo *et al.*, 2013). Desiccation tolerant species of *Selaginella* are of ethnobotanical significance as indigenous groups worldwide use them to treat gastrointestinal disorders (Agra *et al.*, 2008; Kanteh and Norman, 2015; Rojas *et al.*, 1999) and the secondary metabolites created in these plants are

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TABLE 1. Methods for propagating *Selaginella* species reported in the literature.

Species	Purpose	Cultivation medium	Reference
S. kraussiana	microgametophyte cultivation	plaster of Paris in distilled water	Slagg (1932)
S. flabellata *S. pallescens*	megagametophyte cultivation	sterile nutrient agar	Wetmore and Morel (1951)
S. kraussiana	micro/megagametophyte cultivation	soil, wet filter paper	Bierhorst (1964)
S. kraussiana *S. apoda*	sporeling propagation	soil	Webster (1967)
S. kraussiana	megagametophyte cultivation	self-watering filter paper	Robert (1971)
S. martensii var.	intervarietal cross	sterile nutrient agar	Burgeff and Filippi (1957)
S. apoda	intraspecific cross	moist plaster of Paris and distilled water	Bold (1967)
S. kraussiana var.	intervarietal cross	self-watering filter paper apparatus	Webster (1979)
S. apoda	intraspecific cross	soil, water, C-fern agar, water supplemented with nutrients	Schulz *et al.* (2010b)

recognized by pharmacologists for their anticancer properties (reviewed in Gechev *et al.*, 2015).

The nuclear genomes of *Selaginella* are also unique among vascular plants. *Selaginella* nuclear genomes are some of the smallest among green plants with a 1C DNA range of 82–184 Mbp (Obermayer *et al.*, 2002; Little *et al.*, 2007; Baniaga *et al.*, 2016). Unlike other extremely small vascular plant genomes, which are small because of recent reductions (Ibarra-Laclette *et al.*, 2012; Fleischmann *et al.*, 2014; Vu *et al.*, 2015), the small genomes of *Selaginella* appear to be ancestrally small and maintained by the lowest rate of genome size evolution among vascular plants (Baniaga *et al.*, 2016). Perhaps associated with these low rates of genome size evolution is the absence of a paleopolyploidy in the history of *Selaginella* (Banks *et al.*, 2011). Ancient whole genome duplications (WGD) are known from many lineages of vascular plants (Cui *et al.*, 2006; Jiao *et al.*, 2011; Arrigo and Barker, 2012; Kagale *et al.*, 2014; Soltis *et al.*, 2015; Li *et al.*, 2015; Barker *et al.*, 2016), but *Selaginella* is the only genus with a sequenced genome that contains no evidence of any ancient WGD (Banks *et al.*, 2011). Establishing an *in vitro* method of propagation for the resurrection species of *Selaginella* will aid the development of this genus as a model system (Schulz *et al.*, 2010a) to study desiccation tolerance and genome evolution in vascular plants.

Several methods have been applied successfully to propagate gametophytes and sporophytes of *Selaginella* taxa (Table 1). Burgeff and Filippi (1957) cultivated surface-sterilized megaspores and microspores of *S. martensii* Spring vars. together on nutrient agar, flooded the cultures with water to promote fertilization after gametophytes matured, and obtained sporelings

after 30–40 days. Webster (1979) cultivated megaspores and microsporangia of *S. kraussiana* (Kunze) A. Braun separately on filter paper and later placed mature megagametophytes and microgametophytes together to achieve fertilization with sporeling appearance after 30–40 days. More recently, Schulz *et al.* (2010b) used warm nutrient enriched water to propagate gametophytes of *S. apoda* (L.) Spring with high fertilization success. However, there are no reports of propagation for resurrection species of *Selaginella*. In this study, we describe an *in vitro* propagation method for resurrection species of *Selaginella*, a critical tool for research on the biology of desiccation tolerance and evolutionary genetics in the genus.

Materials and Methods

We chose three locally abundant and available resurrection species of Sonoran Desert *Selaginella* for *in vitro* propagation: *Selaginella arizonica* Maxon, *S. eremophila* Maxon, and *S. rupincola* Underw. Mature strobili from both desiccated and hydrated materials of these three species were collected from one population per taxon in the Sonoran Desert and Madrean Sky Islands of Southern Arizona and Southern California. Vouchers for each taxon were deposited at the University of Arizona Herbarium, Tucson, AZ (*S. arizonica* A. Baniaga 739; *S. eremophila* A. Baniaga 739; *S. rupincola* A. Baniaga 740). Mature megasporangia and microsporangia were collected from strobili under a stereoscope, dried at room temperature for three days, and then stored separately by sex in sealed 1.5 mL polypropylene centrifuge tubes in the dark at room temperature until use.

Based on our initial observations for desert *Selaginella*, the microgametophytes germinate and attain sexual maturity much faster than megagametophytes. Due to this developmental difference, our *in vitro* propagation system includes two main steps: the culture of megaspores alone, followed by the addition of microspores to the germinated megaspore cultures. For the megaspore culture, megasporangia were soaked in distilled water for one hour, and dissected on wet filter paper under the stereoscope to isolate individual megaspores. We tested two surface sterilization methods: A) 16.67% bleach solution (sodium hypochlorite) for three minutes as used in C-Fern spore sterilization, or B) 10% bleach solution for seven minutes. Megaspores were surface-sterilized with the two methods, then rinsed five times with sterile distilled water. Megaspores were sown in glass petri dishes (65mm OD x 15mm Height) containing standard C-Fern agar medium (Hickok and Warne, 2004) supplemented with 0.8% (w/v) plant agar, adjusted to pH 6.0. C-Fern medium contains a combination of chelated iron as well as macro- and micronutrient solutions that provide organic compounds for gametophyte growth.

After three weeks of megaspore culture, the co-culture of megaspores and microspores was conducted. Microsporangia were surface-sterilized as described above, and then teased apart with sterile fine-tipped dissecting tools to disperse microspores into sterile distilled water. Afterwards, the

microspore suspension was sown in a petri dish containing germinated megaspores. Another round of surface-sterilized microspores were sown in the same petri dish after two weeks of co-culture to increase the frequency of fertilization. All cultures were maintained under cool-white fluorescent lights (photoperiod 16h light/8h dark) at 25±2°C. Every two to three days, all cultures were observed to record the time of spore germination and gametophyte appearance. After eight weeks of co-culture, the rate of spore germination, spore development into gametophytes, and sporeling formation were recorded. Then sporelings were transferred to a fresh C-Fern agar medium for plantlet cultivation. The spore cultivation of *S. rupincola* was repeated three times, using three replicates every time, *S. arizonica* and *S. eremophila* were repeated four times, using five replicates every time. Each replicate contained 50–60 megaspores from multiple megasporangia, and microspores from ten microsporangia. Variation in the number of replicates was due to differences in the availability of spores for each species.

Results

Initially, we found that mature megaspores and microspores were optimal for successful propagation as immature and small spores did not germinate. Mature megasporangia could be detected by their yellow color and full tetrahedral shape, while mature microsporangia by their amber color and granular texture. Spore surface sterilization was necessary before spores were cultured on the C-Fern agar medium. Initial attempts with unsterilized spores were not successful as fungal contamination appeared, which inhibited spore germination and gametophyte development. We tested two surface sterilization methods: 16.67% bleach solution for three minutes as C-Fern spore sterilization (Hickok and Warne, 2004), or 10% bleach solution for seven minutes. Compared with the C-Fern spore sterilization method, spores sterilized with 10% bleach solution for seven minutes showed higher rate of germination, gametophyte development and fertilization, except for *S.* arizonica which had no fertilization in both methods employed (Table 2). However, there was no difference between the two methods in the time of spore germination or gametophyte appearance (Table 2).

For the three species of *Selaginella*, megaspores germinated after 10–15 days of culture (Table 2). Megaspores became swollen, and opened slightly along the trilete laesurae (Fig. 1a). Microspores germinated much faster, 5–7 days after sowing (Table 2). In our present study, megaspores of these three species showed a relatively low germination rate even when cultured immediately following harvest. When sterilized with 10% bleach solution for seven minutes, more than 20% of *S. eremophila* and *S. rupincola* megaspores germinated. However, only 14.77% of the megaspores of *S. arizonica* germinated. Microspores of all three species had more than twice the germination rate of megaspores, with 42.69–59.49% of all microspores germinating in our cultures.

TABLE 2. Spore germination, gametophyte development, and fertilization of resurrection species of *Selaginella* by surface sterilization method.

Species	Spore	Surface sterilizer[a]	Germination time (days)[b]	Spore germination (%)[c]	First appearance (days)[d]	Gametophyte development (%)[e]	Percent fertilized (%)[f]
Selaginella eremophila	mega	A	15–20	9.00	25–30	3.72	0.00
		B	10–15	29.24	25–30	12.59	12.59
	micro	A	5–7	36.00	10–12	26.94	NA
		B	5–7	59.49	10–12	47.60	NA
Selaginella rupincola	mega	A	15–20	8.22	18–22	3.33	0.00
		B	10–15	20.72	18–22	11.92	11.92
	micro	A	5–7	33.02	10–12	23.13	NA
		B	5–7	42.69	10–12	38.30	NA
Selaginella arizonica	mega	A	15–20	5.05	22–25	2.32	0.00
		B	10–15	14.77	22–25	10.82	0.00
	micro	A	5–7	31.44	10–12	22.11	NA
		B	5–7	46.82	10–12	37.18	NA

[a] Surface sterilizer: A) 16.67% bleach solution for three minutes (C-Fern Method) B) 10% bleach solution for seven minutes
[b] Germination time: the number of days to spore germination
[c] Spore germination: (number of germinating megaspores / total number of megaspores) *100% or (number of germinating microspores / total number of microspores) *100%
[d] First appearance: the number of days to the first appearance of a gametophyte
[e] Gametophyte development: (number of megagametophytes / total number of megaspores) *100% or (number of microgametophytes / total number of microspores) *100%
[f] Percent fertilized: (number of sporelings / total number of megaspores) *100%

Megagametophytes appeared several weeks after sowing. *Selaginella rupincola* megagametophytes were visible after 18–22 days, whereas *S. eremophila* megagametophytes were visible after 25–30 days (Table 2). Megagametophytes were maintained on C-Fern medium for two months or longer (Fig. 1b, 1c). In contrast to megagametophytes, microgametophytes appeared after 10–12 days of culture (Fig. 1d). We also observed microgametophytes on slides under the compound microscope. When microgametophytes were under slight pressure from coverslips, large numbers of sperm cells were released (Fig. 1e). When sterilized with 10% bleach solution for seven minutes, 37.18–47.60% of the microspores of these three species developed into microgametophytes, but only 10.82–12.59% of megaspores developed into megagametophytes.

As the developmental period of megaspores and microspores was different, a two-step method where microspores were sown into established megaspore cultures, was used for propagation. With spores sterilized by 10% bleach solution for seven minutes, the fertilization rate of *S. eremophila* and *S. rupincola* was approximately 12%. Sporelings of *S. eremophila* (Fig. 1f) and *S. rupincola* appeared after 2–5 weeks of co-culture. However, no fertilization or sporelings were observed in *S. arizonica*. We discovered that sporelings should be transferred to the fresh C- Fern medium without water on the surface

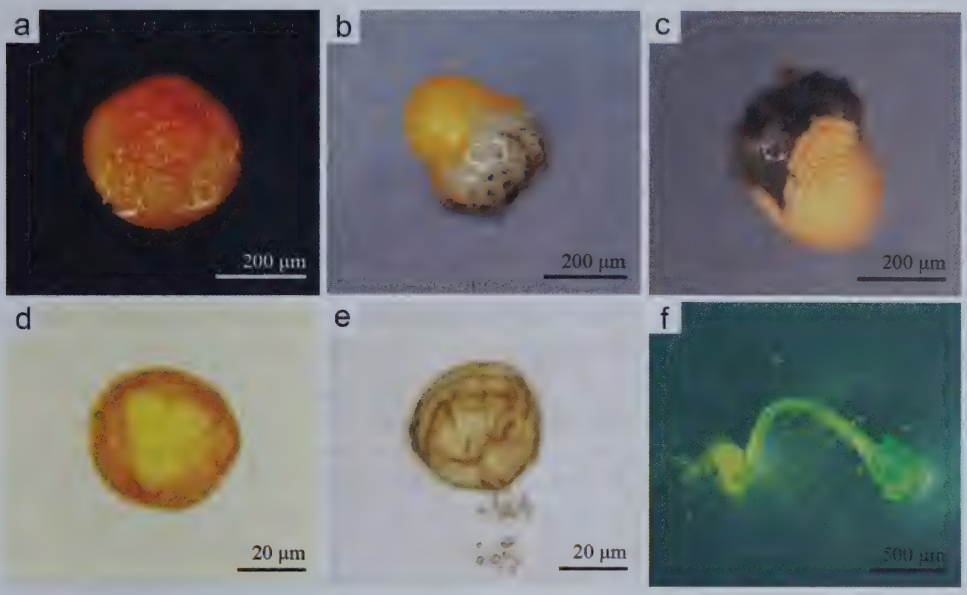

FIG. 1. Gametophytes and sporelings of *Selaginella* **a** Megaspore germination of *S. eremophila*. **b** Megagametophyte of *S. eremophila* after two months of culture. **c** Megagametophyte of *S. rupincola* after two months of culture. d Microgametophyte of *S. arizonica* after ten days of culture. **e** Microgametophyte of *S. arizonica* releasing sperm under slight pressure of cover slip after fifteen days of culture. **f** Sporeling of *S. eremophila*.

of medium. Otherwise, sporelings may develop abnormally with a slender etiolated habit. All sporelings survived after transfer to fresh C- Fern medium.

DISCUSSION

We found that surface-sterilized mature megaspores and microspores were optimal for successful *in vitro* propagation. Similar to previous observations in *Selaginella rupestris* (L.) Spring, small and apparently immature spores failed to germinate (Lyon, 1901). Spore surface sterilization was also necessary for our *in vitro* spore culture. In general, the proper concentration and sterilization time can vary greatly depending on the type of plant material (Fernández and Revilla, 2003), and improper sterilization methods either result in fungal and bacterial contamination, or spore death (Cox *et al.*, 2003; Simabukuro *et al.*, 1998). In our study, we chose bleach as the sterilant. For megaspores and microspores of *S. arizonica*, *S. eremophila*, and *S. rupincola*, surface sterilization with 10% bleach solution for seven minutes had higher germination rates than using the sterilization method for C-Ferns of 16.67% bleach solution for three minutes (Hickok and Warne, 2004). The greater sensitivity of *Selaginella* spores to sodium hypochlorite than C-Ferns is also consistent with reports for *S. apoda* (Schulz *et al.*, 2010b). Such differences in sensitivity between *Selaginella* and leptosporangiate ferns may be attributed to

structural differences in the sporoderm. Although the microspores of some *Selaginella* taxa have a perispore layer, their megaspores appear to lack a perispore layer, which comprises a majority of the sporoderm in many leptosporangiate ferns (Tryon and Lugardon, 1991).

Megaspores of *Selaginella* germinated after 10–15 days of culture (Table 2), which is similar to megaspore germination of *Selaginella kraussiana* and its varieties (Webster, 1979). According to Robert (1971), megaspores of *S. kraussiana* exhibited 90% germination if cultured immediately following harvest and less than 50% if culture was delayed for three months. However, in our study, megaspores of these three species showed a much lower germination rate even when cultured immediately following harvest. Less desiccation tolerant species of *Selaginella*, such as *S. kraussiana* and its varieties, had a 60–90% successful fertilization rate (Webster, 1979), and *S. apoda* had a 60–95% successful fertilization rate (Schulz *et al.* 2010b). Compared with the desiccation sensitive taxa, the much more desiccation tolerant taxa exhibited a lower fertilization rate of approximately 12% in *S. eremophila* and *S. rupincola*, and no fertilization in *S. arizonica* (Table 2). At present, we cannot determine the reason for the low germination and fertilization rates. The low rates may be due to differences related to their unique desiccation tolerance or more general reasons concerning surface sterilization and culturing media. In some fern species the surface sterilization method and factors of medium, such as pH, nutrients, and sucrose concentration can greatly influence spore germination and gametophyte development (Cox *et al.*, 2003; Wu *et al.*, 2010). For the three desiccation tolerant species of *Selaginella*, we suggest future work on improving the surface sterilization method as well as the culturing medium in order to increase gametophyte germination and fertilization rates. Although there may be future improvements to these culturing methods, the technique presented here provides successful *in vitro* propagation for previously uncultured species of *Selaginella*.

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COVER CAPTION: Lower surface of pinnae of *Adiantum rivularis*. Photo by Vincent Pelletier.